University of Alberta

Molecular Characterization of *S10-spc-α* Ribosomal Protein Gene Operons and RNA Polymerase α Subunit of *Chlamydia trachomatis*

By

Lijie Gu ©

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Molecular Characterization of *S10-spc-α* Ribosomal Protein Gene Operons and RNA Polymerase α Subunit of *Chlamydia trachomatis*

submitted by Lijie Gu in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences - Pediatrics.

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Dedication

To my parents, for their support and understanding.

To my wife Jiaping Shi for her love, encouragement and patience.

To my daughter Donia.

Abstract

I have sequenced and characterized a 8350-bp segment of *Chlamydia trachomatis* chromosomal DNA that shows homology to the *Escherichia coli spc-α* and distal region of the *S10* ribosomal protein (r-protein) gene operons. Its sequence revealed a high degree of nucleotide and operon context conservation with the *E. coli* r-protein genes. The *C. trachomatis spc-α* operons contain the genes for the r-proteins L14, L24, L5, S8, L6, L18, S5, L15, SecY, S13, S11, α and L17 along with the genes for r-proteins L16, L29 and S17 of the *S10* operon. The *S10* and *spc* operons are separated by a 16-bp intergenic region which contains no transcription signals. However, a putative promoter for the transcription of the *spc* operon was found 162 nucleotides upstream of the CtrL14e start site; it revealed significant homology to the *E. coli* consensus promoter sequences. A 56-bp intergenic region was found between *secY*, the last gene of the *spc* operon, and S13, the first gene of α operon. While primer extension experiments identified a putative promoter just upstream of the α operon, computer searches located a rho-independent terminator distal to the αORF.

Characterization of the gene(s) involved in the process of protein translocation is important in understanding their structure function relationship. However, little is known about the signals that govern chlamydial gene expression and translocation. The gene product of the *C. trachomatis secY* is composed of 457 amino acids with a calculated molecular weight of 50,195 daltons. This

amino acid sequence bears 27.4% and 35.7% identities to *E. coli* and *B. subtilis* SecY proteins respectively. The distribution of hydrophobic amino acids in the *C. trachomatis secY* gene product is suggestive of it being an integral membrane protein with ten transmembrane segments, the second, third and seventh membrane segments sharing greater than 45% identity with *E. coli* SecY protein. Further, the expressed chlamydial SecY protein exhibits different molecular sizes based on its mobility in SDS-PAGE of varying concentrations. These results suggest that despite evolutionary differences, eubacteria share a similar protein export apparatus.

Immunoblot analysis identified α protein expression at 12 hours post infection in *Chlamydia*-infected HeLa cells. When overexpressed in *E. coli*, the chlamydial α subunit was incorporated into *E. coli* RNA polymerase (RNAP). In contrast, a deletion lacking a portion of the amino terminus of the chlamydial α subunit failed to copurify with *E. coli* RNAP. The cross reactivity of anti chlamydial α subunit antibodies with *E. coli* RNAP α subunit as well as incorporation of chlamydial α subunit protein into *E. coli* RNAP holoenzyme suggests the presence of structural and antigenic conservation among α subunits from distantly related genera. The chlamydial-α-incorporated *E. coli* RNAP (chimeric RNAP) and overexpressed free chlamydial α subunit protein were purified by affinity column and glycerol gradient centrifugation. The purified chimeric *E. coli* RNAP is fully functional and has the ability to transcribe the chlamydial MOMP gene *in vitro*. The pure chlamydial α subunit protein was demonstrated to specifically bind to the DNA fragment containing the MOMP promoter region. The chlamydial *S10-spc-α* operons were mapped in the region between the genes for β, β' and the gene for σ^{66} in the chromosome.

In summary, the work presented provides some important insight on chlamydial ribosomal protein genes, genetic and biochemical studies of chlamydial SecY protein and RNAP α subunit. Functional chimeric RNAP presents a unique approach to purify RNAP and to perform *in vitro* chlamydial genetic studies.

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List of Abbreviations

Bis	N,N'-methylene-bis-acrylamide
BSA	bovine serum albumin
Ci	Curie
CPM	counts per minute
CsCl	cesium chloride
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EB	elementary body
EDTA	ethylene diamine tetraacetate
EtBr	ethidium bromide
FCS	fetal calf serum
FSB	final sample buffer
GST	glutathione-S-transferase
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton
kV	kilovolt
LGV	lymphogranuloma venereum
MAb	monoclonal antibody

MEM	minimal essential medium
min	minute
MOMP	major outer membrane protein
NP40	nonidet-P40
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RB	reticulate body
RF	replicative form
RNA	ribonucleic acid
RNAP	RNA polymerase
RNase	ribonuclease
SDS	sodium dodecyl sulfate
SSC	"standard saline citrate"= 0.15M NaCl+0.015M Nacitrate, pH 7.0
TCA	trichloroacetic acid
TE	tris-EDTA
TEB	tris-EDTA-borate
TEMED	N,N,N',N'-tetramethylethylene diamine
TES	tris-EDTA-sodium chloride
Tris	tris(hydroxymethyl)-aminomethane
tRNA	transfer RNA

TSN	tris saline-NP40
UV	ultraviolet
x g	times gravitational force
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YT	yeast-tryptone medium

I. Introduction

I.1 *Chlamydia* and its characteristics

Chlamydiae are obligate intracellular parasites which evolve within host eukaryotic cells. Once classified as viruses, they are now considered specialized bacteria that possess discrete cell walls, contain both DNA and RNA, multiply by binary fission and are susceptible to antimicrobials (Moulder, 1988). Chlamydiae differ from conventional bacteria in their intracellular nature, small size, longer generation time and a requirement for an exogenous ATP supply. Chlamydiae are amongst the most successful human/animal pathogens and are linked to an expanding spectrum of human diseases, constituting an important public health problem. *Chlamydia trachomatis* is responsible for 350 million cases of trachoma in the developing countries and is the leading preventable cause of blindness worldwide. It is estimated that more than 3 million new genital infections occur each year in North America (Schachter, 1988). Coupled to these statistics is the observation that sexually acquired lesions and inflammation can facilitate HIV transmission and infected individuals are at three- to five- fold increased risk of acquiring HIV if exposed to that virus.

The order Chlamydiales consists of one family, Chlamydiaceae, with one genus, *Chlamydia* (Moulder, 1988). The genus *Chlamydia* is composed of three species: *Chlamydia trachomatis*, *Chlamydia psittaci*, and *Chlamydia pneumoniae*. There are 15 serovars within the species *C. trachomatis* including serovars L_1-L_3 that are responsible for lymphogranuloma venereum, serovars A-C that are responsible for trachoma and serovars D-K that cause genital tract infections. Genus-specific lipopolysaccharides and cell envelope protein antigens have been used routinely to serotype chlamydial species. The antigenic differences between the various subtypes of *C. trachomatis* appear to reside mainly in its major outer membrane protein (MOMP), which constitutes roughly 60% of the total outer membrane. *C. psittaci*, however, is much more heterogeneous and no attempt has been made to subdivide it. *C. pneumoniae* is a relatively new species containing the TWAR (TaiWan Acute Respiratory disease) strains, which have been isolated from acute respiratory infections in humans (Grayston, 1989). DNA hybridization between the chlamydial species revealed less than 10% homology among the three species, which is well below the 20% cutoff point suggested for members of the same genus (Johnson, 1984). Low DNA homology between *Chlamydia* species may suggest that they have diverged long ago or have different ancestral lineage (Kingsbury and Weiss, 1968). Chlamydiae can also be differentiated by other characteristics e.g., *C. trachomatis* strains are sensitive to sulfonamide and synthesize large amounts of glycogen which surrounds the inclusion bodies, while *C. psittaci* and *C. pneumoniae* strains are resistant to sulfonamide and contain no glycogen (Moulder, 1988). Despite the lack of DNA relatedness and differential response to sulfonamides these three species are clearly related by their common developmental life cycle, common antigens and similar biological and metabolic activities, justifying placing them in the same genus (Ward, 1983).

Chlamydiae have evolved a complex and unique life cycle which involves two forms: the extracellular, small (0.2-0.3 μm), rigid, infectious elementary bodies (EB) and the intracellular, large (1 μm), fragile, reproductive reticulate body (RB) (Higashi, 1965). The two life forms are morphologically and functionally distinct. While EBs are small and possess a discrete and electron dense nucleoid, RBs are large and contain an evenly dispersed cytoplasm (Hatch et al. 1984; Tamura and Manire, 1967). These characteristics are believed to render EBs incapable of participating in the transcription and replication process as compared to the RB form, whose nucleoid is quite compatible for replication. In addition, EBs usually have a very rigid cell well with cysteine-rich proteins on the surface cross linked by extensive disulfide bonds, in contrast to RBs that appear pleomorphic and do not contain any disulfide cross linked proteins (Hatch et al. 1984; Caldwell et al. 1981; Bavoil et al. 1984; Hackstadt et al. 1985). Functionally, EBs exhibit cytotoxicity for mice (Christoffersen and Manire, 1969), and macrophages in tissue culture (Brownridge and Wyrick, 1979) and contain hemagglutinin (Tamura and Manire, 1974), while RBs are involved in macromolecular synthesis and active transport of ATP, GTP and lysine from host cells (Hatch et al. 1982).

The infection is initiated by attachment of EB to its host cell membrane through a heparin sulfate like glycosaminoglycan component produced by the microorganism. The attached EB are subsequently internalized and established in the host cell by processes involving rearrangement of F-actin filaments and tyrosine phosphorylation of host cell proteins (Stephens, 1994). Metabolic changes that are incompletely understood lead the EB to reorganize within about 1-8 hours into the large RB. Using the ATP-generating capacity of the host cell, RBs divide by binary fission within the endocytic vacuole. The newly formed organisms occupy much of the infected host cell, producing a cytoplasmic inclusion body characteristic of *Chlamydia* infection. Electron micrographs show that RBs have many more ribosomes than EBs, as would be expected of growing and dividing cells. The life cycle is completed with the reorganization of RB into EB leading to DNA condensation and disulfide cross linking of EB outer membrane protein (Ward, 1988). The life cycle takes anywhere from 48-72 hours depending upon the strain, culminating in completely deranged host cellular and subcellular material (Todd and Storz, 1975).

The genetics of chlamydial regulation is largely undefined, mainly due to the lack of any convenient system for gene transfer, and also due to the paucity of information about the signals and machinery that govern gene expression. The chlamydial genome (comprising a closed double stranded chromosome and 7.4 kb cryptic plasmid) is approximately 1.045×10^6 nucleotide base pairs and is substantially smaller than that of most bacteria and Rickettsiae, although larger than some mycoplasmas and large DNA viruses (Birkelund and Stephens, 1992; Sarov and Becker, 1969; Lovett et al. 1980). The small size of chlamydial EB is likely a reflection of its adaptation to an intracellular environment and is probably due to the lack of a need to encode enzymes involved in energy metabolism.

The recent application of molecular genetic techniques to chlamydial research has provided insight into the regulation of a few genes. Several groups have cloned and sequenced some of the chlamydial genes that play important structural and functional roles, among them the genes for chlamydial histone H1-like proteins whose expression is closely associated with differentiation of RB to EB (Tao et al. 1991; Hackstadt et al. 1991; Perara et al. 1992; Brickman et al. 1993). These histone H1 homologs appear to play a major role in controlling the chlamydial life cycle through their ability to modify DNA structure (Barry et al. 1992; Christiansen et al. 1993; Barry et al. 1993; Pedersen et al. 1994). Other important genes include operons whose expression is coordinately regulated. Examples of coordinately controlled operons in *Chlamydia* are the 16S and 23S rRNA operon (Engel and Ganem, 1987), 15 and 60 kDa cysteine-rich outer membrane protein genes (Clarke et al. 1988; Everett and Hatch, 1991; Morrison et al. 1989), *gro*ES and *gro*EL heat shock protein gene homologs from *C. trachomatis* and *C. psittaci* (Engel et al. 1990b). In addition, sequences upstream and downstream of chlamydial β subunit of RNAP revealed homologies to ribosomal protein (r-protein) L7/L12 and β' subunit homologs of *E. coli* (Engel et al. 1990a). Together, these findings indicate that part, if not all, of the structure is conserved in the r-protein gene operon comprising the RNAP subunits.

I.2 Ribosomes and ribosomal protein genes

Ribosomes are the molecular machines that coordinate the interplay of tRNAs, mRNA and proteins in the complex process of protein synthesis. The main aim of intact ribosomes is to polymerize amino acids into proteins. An *E. coli* ribosome is a ribonucleoprotein particle with a mass of about 2700 kDa, a diameter of approximately 200 Å, and a sedimentation coefficient of 70S. It can be dissociated into a large subunit (50S or L subunit) and a small subunit (30S or S subunit) (Lake, 1985). These subunits can be further split into their constituent proteins and rRNAs. The 30S subunit contains 21 different r-proteins (labeled S1 to S21) and a 16S rRNA molecule (Lindahl and Zengel, 1982; Lake, 1985). The 50S subunit contains 34 different r-proteins (labeled L1 to L34) and two rRNA molecules, a 23S and a 5S rRNA species. A ribosome contains one copy of each RNA molecule, two of L7 and L12, and one of each other protein (Lindahl and Zengel, 1982; Lake, 1985).

With the development of the electron microscope Hall and Slayter (1959) were able to show that a characteristic feature of the visible ribosomal projection was a groove dividing the ribosome into two unequal parts. This subdivision reflected the fact that the ribosome consists of two separable subparticles, or ribosomal subunits. The biochemical components of the ribosomes were firmly established by Kurland (1960) who demonstrated that the observed two major species of rRNAs, 16s and 23s RNAs in the case of bacterial rRNAs, are really two distinct species, each representing covalently linked unique and homogeneous molecules without any subunit structures. While the 30S ribosomal subunit plays an important role in initiation of protein synthesis by its mRNA-binding ability, the 50S subunit alone can carry out the peptidyl-transferase reaction (Nomura et al. 1967). Substantial evidence for potential roles of r-protein in protein-induced conformation changes in rRNA is accumulating. Ribosomal proteins may stabilize RNA structure, facilitate required RNA structural transitions and provide needed positive charge or other functional groups (Draper, 1990).

The genes for all 55 r-proteins are organized into 19 different operons throughout *E. coli* chromosome (Lindahl and Zengel, 1986; Nomura et al. 1984). In *E. coli* three of the 19 r-protein gene operons viz., *S10*, *spc* and α lie adjacent to each other and are located at the 72 minute position of the chromosome. The *E. coli S10* operon sequentially encodes S10, L3, L4, L23, L22, S19, S3, L16, L29 and S17 while the *spc* operon encodes the genes for r-proteins L14, L24, L5, S14, S8, L6, L18, S5, L30 and L15. In addition, the *spc* operon contains genes for L36, also called X, and *secY* involved in protein export machinery (Cerretti et al. 1983; Ueguchi et al. 1989).

The intergenic region between the *S10* and *spc* operons in *E. coli* is 170 bp while that between the *spc* and α operons is 150 bp. An independent promoter for each of the *S10*, *spc* and α operons exists in these intergenic regions. The *spc* operon genes are autogenously regulated by a translational coupling mechanism whereby the S8 gene product binds to the target site on L5 mRNA leading to repression of L5 translation, and consequently of the downstream mRNA of the *spc* operon (Cerretti et al. 1988; Mattheakis and Nomura, 1988). In contrast, feedback regulation of the *S10* operon involves both translational repression by L4 and transcriptional repression through an attenuation mechanism by the same repressor (Lindahl et al. 1983; Freedman et al. 1987). Feedback regulation of r-protein gene expression in *E. coli* is due to the structural homology of rRNA and r-protein mRNA in competition for the binding of repressor r-proteins (Nomura et al. 1980).

Downstream of the *spc* operon are located the genes of the α operon that encode four r-proteins: S13, S11, S4 and L17, in addition to, surprisingly, the RNAP α subunit. 70% of the r-protein genes in the α operon are transcribed as read through from the promoter for the *spc* operon and only 30% of transcription is initiated from its own promoter to regulate components of ribosome synthesis (Lindahl et al. 1990). Expression of these four r-protein genes in the *E. coli* α operon is regulated at the level of translation by the binding of S4 to the α mRNA. (Yates et al. 1980).

Earlier attempts at characterizing the rRNA from chlamydial species have met with some success. Tamura and Iwanagal (1965) identified 21S, 16S and 4S rRNA fractions in *C. psittaci*: of these, 21S and 16S were the predominant forms in RBs, whereas 4S predominated in the EBs. Sarov and Becker (1968) found similar rRNA species in *C. trachomatis*. On the basis of their 16S rRNA gene sequence, chlamydiae have been identified as eubacterial in origin, related peripherally to planctomyces (Weisburg et al. 1986). Further, Palmer et. al. (1986) found that chlamydial 16S rRNA genes are present in two copies on the chromosome. Recently, a few chlamydial ribosomal protein genes have been cloned and characterized that include the genes for r-protein L6 (Gray et al. 1991), S7/S12 with elongation factors EF-G and EF-Tu in the *str* operon (Wagar and Pang, 1992), S18 (Engel et al. 1990b), and L7/L12 along with RNAP β, β' subunits in the same operon (Engel et al. 1990a).

I.3 Protein secretion and SecY protein

Proteins destined to be localized in the periplasm or the outer membrane of *E. coli* are synthesized as precursors comprising the mature proteins with a signal sequence at their N-termini. Some of these proteins are then moved via translocation machinery in the inner membrane to be integrated into the inner membrane, transported across to the periplasm, integrated into the outer membrane, or exported from the cell. Cleavage by a leader peptidase occurs at the periplasmic face of the inner membrane, then the mature protein moves to its final destination. Translocation of these precursors across the cytoplasmic membrane in prokaryotes is catalyzed by machinery comprising the Sec proteins. Interestingly, the gene encoding the central component of protein translocation apparatus, the SecY protein, is located in the distal region of the *spc* r-protein gene operon (Cerretti et al. 1983).

The process involved in protein translocation of a newly synthesized polypeptide across cytoplasmic membranes is highly complex and multifactorial. The factors in *E. coli* identified so far include: SecA (Lill et al. 1989; Cunningham et al. 1989), SecB (Collier et al. 1988), SecD (Gardel et al. 1987), SecE (Schatz et al. 1989), SecF (Gardel et al. 1990), SecG (Brundage et al. 1990; Douville et al. 1994) and SecY (Akiyama and Ito, 1987). Five out of seven genes, *secD*, *secE*, *secF*, *secG* and *secY*, encode integral membrane proteins.

SecB is a chaperone protein (Collier et al. 1988). Several chaperones, such as heat shock protein and GroEL, (Jungnickel et al. 1994; Lecker et al. 1989) have been shown to increase the efficiency of bacterial protein export by preventing premature folding. SecB plays the major role in promoting export, probably because it has two functions. It can behave as a chaperone and bind to a nascent protein; and it has an affinity for the protein SecA (Altman et al. 1990). In its function as a chaperone, SecB binds to a precursor protein to retard folding, but it is unable to reverse the change in structure of a folded protein (Weiss et al. 1988). Thus it does not function as an unfolding factor; its role is strictly to inhibit improper folding of the newly synthesized protein. The affinity of SecB for SecA allows it to target a precursor protein to the membrane.

SecA is a large peripheral membrane protein which associates with the membrane by virtue of its affinity for acidic lipids and for the protein SecY (Akita et al. 1990; Matsuyama et al. 1990b; Lill et al. 1990; Fandl et al. 1988; Lill et al. 1989). SecA recognizes both SecB and the precursor protein that it chaperones; probably features of the mature protein sequence as well as its leader sequence are required for recognition (Akita et al. 1990; Matsuyama et al. 1990b). SecA has an ATPase activity which depends on binding to lipids, SecY and a precursor protein. The ATPase functions in a cyclical manner during translocation. After SecA binds a precursor protein, it binds ATP, and about 20 amino acids are translocated through the membrane (Breukink et al. 1992; Ulbrandt et al. 1992). Hydrolysis of ATP is required to release the precursor from SecA.

The gene *secY* was first identified as *prlA* by suppresser mutants that restored export of a protein with a defective signal sequence (Shultz et al. 1982; Emr and Bassford, 1982), suggesting that the gene product interacts directly with signal sequences. Later studies showed that the product of the gene complemented the pleiotropic protein export mutant obtained by localized mutagenesis of the *spc* ribosomal protein operon within open reading frame "Y" (Cerretti et al. 1983; Ito, 1990; Shiba et al. 1984). The product of the *secY* gene is a hydrophobic and highly basic protein of about 49 kDa. It contains 10 transmembrane segments, six cytoplasmic domains and five periplasmic regions (Akiyama and Ito, 1987). The SecY protein is thought to form a channel through which presecretory proteins are translocated across the membrane. Together with SecE and SecG, SecY forms a central translocase for protein export (Brundage et al. 1990; Douville et al. 1994). The estimated cellular concentration of SecY is about 300 molecules per cell in *E. coli* (Ito, 1990). Recently, SecY homologs have been reported in the gram positive eubacteria *Bacillus subtilis* (Nakamura et al. 1990; Suh et al. 1990) and *Lactococcus lactis* (Koivula et al. 1991), the archaebacterium *Cryptomonas phi* (Douglas, 1992) and yeast (Stirling and Hewitt, 1992).

SecE, a 14 kDa protein, is thought to span the membrane three times (Schatz et al. 1989). Both genetic (Schatz et al. 1991) and biochemical (Nishiyama et al. 1992) studies revealed that the SecE function exists in the third transmembrane segment and preceding cytoplasmic region. SecY and SecE most likely function as a complex since SecY and SecE can be isolated as a complex (Brundage et al. 1990), and overproduction of SecY is possible when SecE is overproduced at the same time (Matsuyama et al. 1990a). The isolated SecY/SecE complex often contains another protein termed Band I (Brundage et al. 1990; Brundage et al. 1992). The role of Band I in the protein translocation was not clear until recently since the translocation machinery can be reconstituted with highly purified SecY, SecE and SecA, without other proteins (Akimaru et al. 1991). The activity of proteoliposomes reconstituted with the three Sec proteins was considerably lower than that of membrane vesicles. SecG, formerly termed P12, was found as a new component which stimulates the reconstituted activity (Nishiyama et al. 1993). Disruption of this gene impaired the protein translocation *in vivo* (Hanada et al. 1994), indicating that SecG is directly involved in protein translocation in *E. coli*. Immunochemical studies involving an antibody raised against a partial sequence of SecG revealed recently that SecG is identical to Band I (Douville et al. 1994). SecG also plays a critical role in cold stress (Nishiyama et al. 1994) and enhances the translocation activity of the entire protein translocation apparatus after the step of SecA targeting to SecY-SecE (Hanada et al. 1994).

The functions of the *secD* and *secF* gene products are not yet fully elucidated. The products of the *secD* and *secF* locus probably regulate the channel assembly-disassembly reaction by modulating the SecA insertion-deinsertion step (Kim et al. 1994).

Genetic techniques have been applied to identify genes that encode components of the translocation apparatus mainly in *E. coli*. Central to the process of protein translocation is the role of the *secY* gene product which acts as a translocator through which proteins cross the inner membranes (Bieker and Silhavy, 1990; Brundage et al. 1990) although the process involved in protein export of a newly synthesized polypeptide across the cytoplasmic membrane is highly complex and multifactorial. Factors identified to date include: a cytosolic chaperone protein, SecB (to prevent preprotein aggregation and premature folding) (Collier et al. 1988; Weiss et al. 1988), a preprotein translocase comprising a peripheral translocation ATPase protein, SecA (exposed to the periplasmic surface of the inner membrane in its active state to supply energy from hydrolyzing ATP) (Kim et al. 1994; Chen and Tai, 1985; Lill et al. 1989) and a membrane-embedded central translocation channel protein complex of SecY, SecE and SecG (translocase) (Nishiyama et al. 1994; Bieker and Silhavy, 1990; Brundage et al. 1992). Further studies have also invoked the role of SecD and SecF in the late stage translocation process (Gardel et al. 1987; Gardel et al. 1990; Sagara et al. 1994). Translocation is energy dependent, utilizing ATP and the proton-motive force that exists across the inner membrane. Recently, it has been demonstrated that preprotein targeting to the plasma membrane is mediated by the binding cascade of SecB to SecA to SecY/E/G (Hartl et al. 1990; Kim et al. 1994). These studies were further substantiated by reconstitution of the protein translocation system using purified *E. coli* SecY, SecE, SecG and SecA gene products (Hanada et al. 1994).

Characterization of the gene(s) involved in the process of protein translocation is important in understanding their structure function relationship. However, little is known about the signals that govern chlamydial gene expression and protein translocation. To date, no cloned chlamydial *sec* genes and protein translocation mechanisms have been reported. The mechanism and pathway of protein translocation across the chlamydial cytoplasmic membrane and how exported proteins are processed within the two different forms of the chlamydial life cycle are still unclear.

I.4 RNAP and RNAP α subunit

DNA-dependent RNAP is the key enzyme for the transcription of genetic information. The structure and molecular properties of RNAP are intimately linked to the structure of the entire transcription apparatus of the cell. Just as the ribosome is the central element in translation and carries out the catalytic coupling of amino acids into peptide linkage, RNAP is responsible for the actual coupling of nucleotides to give the nascent RNA chain. The structure of the *E. coli* DNA-dependent RNAP was first reported in 1969 (Burgess, 1969). The *E. coli* RNAP has four major subunits, designated β', β, σ and α. The protomeric form has the structure of $\alpha_2\beta'\beta\sigma$ (holoenzyme) (Burgess and Travers, 1970; Berg and Chamberlin, 1970). Molecular weight values estimated from polyacrylamide gels after denaturation with SDS vary somewhat in different reports. The best current estimates are $\beta'=160{,}000$, $\beta=150{,}000$, $\sigma=86{,}000$ and $\alpha=40{,}000$.

The holoenzyme of RNAP ($\alpha_2\beta\beta'\sigma$) can be separated into two components, the core enzyme ($\alpha_2\beta\beta'$) and the sigma factor (σ). The names reflect the fact that only the holoenzyme can initiate transcription; but then σ is released, leaving the core enzyme to undertake elongation (Helmann and Chamberlin, 1988). Thus the core enzyme has the ability to synthesize RNA on a DNA template, but cannot initiate transcription at the proper sites. The function of the α subunit in RNAP is to associate β and β' subunits to form the core enzyme, to bind some specific promoter regions and to enhance RNA transcription through protein-protein contact (Igarashi and Ishihama, 1991; Ishihama, 1992). The β' subunit is able to bind DNA template nonspecifically. The β subunit is mostly involved in catalysis of RNA synthesis (initiation and elongation), binding of ribonucleoside triphosphate substrates and binding of product RNA. The function of σ is to ensure that a bacterial RNAP binds to DNA more stably only at specific promoters and confers the ability to recognize specific binding sites to stimulate initiation of RNA synthesis (Ishihama, 1981).

Bacterial RNAPs from different species appear to be closely related in subunit structure (Burgess, 1976). The structure of RNAP from other Gram-negative bacteria resembles that of the *E. coli* enzyme, although the molecular weight values obtained by gel electrophoresis for σ and α are slightly higher (Herzfeld and Zillig, 1971). The subunits of RNAP isolated from various *Bacillus* species also resemble those of the *E. coli* enzyme except that the molecular weight estimated for the subunit resembling the *E. coli* σ subunit is much lower. Genetic and biochemical evidence that β', β, σ and α are functional subunits of the DNA-dependent RNAP is also well documented. Mutants of *E. coli* resistant to rifampicin have an RNAP altered in the β subunit (Lowder and Johnson, 1987). Certain temperature-sensitive mutants of *E. coli* RNAP are altered in the β' subunit (Panny et al. 1974).

The functions of the *E. coli* RNAP α subunit have recently been studied extensively (Russo and Silhavy, 1992; Ishihama, 1993; Ebright and Busby, 1995). The RNAP α subunit performs at least three critical functions: i) initiating for RNAP assembly; 2) participating in promoter recognition by sequence-specific protein-DNA interaction, and 3) regulating transcription by protein-protein interaction with a large set of transcriptional activators, including catabolite gene activator protein (CAP) (Igarashi and Ishihama, 1991), phage lambda host factor (Giladi et al. 1992), OxyR protein (Tao et al. 1993). Ishihama (1992) demonstrated that the N-terminal two-thirds of the α subunit of *E. coli* RNAP plays an essential role in the initiation of subunit assembly by gathering two large subunits, β and β', together into a core-enzyme complex, while the C-terminal region of the RNAP α subunit is involved in protein-protein contact in positive control of transcription (Igarashi and Ishihama, 1991; Tao et al. 1993). Recently, purified α subunit was demonstrated to bind specifically to the AT-rich DNA sequence located upstream of the -10, -35 region of the *E. coli* ribosomal RNA promoter *rrnB* P1 (UP element), hence to stimulate transcription, confirming that the α subunit acts directly in promoter recognition (Ross et al. 1993).

Transcription studies in *Chlamydia* are still in their infancy. Using the polymerase chain reaction (PCR) technique, chlamydial RNAP β, β' and σ homologs were identified from *C. trachomatis* strain MoPn (Engel et al. 1990a; Engel and Ganem, 1990). The chlamydial RNAP β and β' subunits have similar molecular weights to those of *E. coli* RNAP β and β' subunits (Engel and Ganem, 1990). The chlamydial σ^{70} homologs isolated from *C. trachomatis* human strain and *C. psittaci* strain 6BC have molecular weights of 66,000 (chlamydial σ^{66}) (Koehler et al. 1990; Douglas et al. 1994). Despite the homology between chlamydial σ^{66} and *E. coli* σ^{70}, recent genetic studies have shown that the sequence requirement for chlamydial promoters differs from that of *E. coli* and chlamydial RNAP can tolerate considerably more variation at the -10 and -35 regions (Mathews and Sriprakash, 1994). Purification of RNAP from *Chlamydia* represents a difficult process because of the limitations imposed by very small amounts of starting material. Despite such limitations *in vitro* host-free RNA synthesis in *Chlamydia* has recently been established by Hatch's group (Crenshaw et al. 1990; Mathews et al. 1993), demonstrating that radiolabeled GTP could be incorporated into RNA in host-free *C. trachomatis* serovar L_2 organisms and the pattern of transcription by host-free Chlamydiae is similar to the pattern of *in vivo* transcription at the time of isolation of Chlamydiae. In addition, this group also showed that *in vitro* transcription was enhanced ten fold through addition of cloned and overexpressed chlamydial σ^{66}, suggesting unsaturation of chlamydial RNAP with σ^{66} (Douglas et al. 1994).

II. Materials and Methods

II.1 Tissue culture and *Chlamydia* infection

C. trachomatis serovar L_2 (L_2/434/Bu) was grown in HeLa 229 cells as described by Kuo et al (1977). Briefly, HeLa cells were grown in defined minimal essential medium (MEM, GIBCO BRL Life Technology) containing 7% fetal calf serum (FCS), 0.2% $NaHCO_3$, 2 mM L-glutamine and 1 µg/ml gentamicin in an incubator supplied with 5% CO_2. Chlamydial infection was usually carried out after the HeLa cells were confluent. For each flask (175 cm^2), 50 µg of EBs were diluted in 2 ml of 1 X HBSS, briefly sonicated, then used to infect HeLa cells. The flasks for infection were incubated at room temperature for 2 hours, with gentle rocking every 20 minutes. After a 2-hour incubation period, unbound EBs were removed by washing with 1 X HBSS. The infected HeLa cells were supplied with fresh culture medium as above plus 1 µg/ml of cycloheximide to inhibit protein synthesis of eukaryotic cells, and the flasks were returned to the incubator at 37°C with 5% CO_2.

II.2 Isolation of *Chlamydia* EBs and RBs

EBs were harvested at 48 hours post infection by centrifugation and purified as reported earlier (Gray et al. 1991). Briefly, the culture medium of infected HeLa cells was poured off. The cells were washed with 1 X HBSS and removed from the wall of flasks with glass beads. The suspended HeLa cells were pipetted into centrifuge tubes and sonicated briefly at a setting of 6 (Sonicator^{TM} Model W-

220F, Heat Systems-Ultrasonics Inc., Plainview, New York) for 30 seconds on ice. The sonicated cells were centrifuged at 500 X g for 15 min at 4°C. The supernatant was layered over 10 ml of 35% renografin and subjected to centrifugation at 43,000 X g for 60 min at 4°C. The pellet was resuspended in TES (10 mM N-tris(Hydroxymethyl)methyl-2-aminoethane-sulfonic acid, 0.87% NaCl, pH 7.4), layered over a discontinuous renografin gradient (7 ml 52%, 10 ml 44% and 15 ml 40% of renografin) and centrifuged in an SW28 rotor at 19,000 rpm (50,000 X g) for 60 min at 4°C. EBs were collected from the interface between the 52% and 44% layers of renografin. The collected EBs were washed with 10 volumes of TES once and the centrifugation was repeated. Finally, purified EBs were resuspended in SPG (see Appendix) and stored at -70°C.

Chlamydial RBs were usually harvested at 18 to 24 hours post infection. The supernatant was layered over 35% renografin and spun at 13,000 rpm (J2-21 rotor, 20,000 X g) for 60 min after sonication and centrifugation at 500 X g for 15 min at 4°C. The resultant pellet was resuspended in TES and layered over a continuous renografin gradient (35%-65%) and centrifuged at 17,000 rpm in an SW28 rotor (40,000 X g) for 90 min at 4°C. A continuous renografin gradient (35%-65%) was prepared by freezing 50% renografin in TES at -20°C and thawing it slowly at 4°C overnight before use. Pure RBs were collected from the band in the middle of the gradient and washed with TES. Finally, purified RBs were resuspended in SPG and stored at -70°C.

II.3 Other bacterial strains, plasmids and bacteriophages

E. coli strains XL-1 Blue, NM522 and DH5α' were used as hosts for pBluescript, pUC and bacteriophage M13, whereas BL-21 (DE3) or BL-21 (DE3) LysE strain was used for protein expression of clones in either pBluescript or pT7-7 vectors. *E. coli* strain BNN103 was used as a host for the fusion protein expression vector pGEX-3X. The strains XL-1 and DH5α' were grown on minimal medium to maintain the F' episome. BL-21 (DE3) LysE was grown with selection by chloramphenicol to maintain the plasmid containing the *lysE* gene. Other strains were grown on LB medium. *E. coli secY*ts24 mutant IQ85 (Shiba et al. 1984) was a gift from Dr. K. Ito at Tokyo University, Japan.

The plasmids pBluescript SK+ and the pUC series were used for cloning, recombinant DNA amplification and double stranded DNA sequencing. The bacteriophage M13 series was used for single-stranded DNA sequencing and the plasmid pT7-7 for protein expression. pGEX-3X was used to generate fusion proteins.

Competent cells for chemical transformation were prepared as follows: a culture inoculated with a 1 to 100 dilution of overnight culture was grown for about 2 hours (OD_{600}: 0.3-0.4). The culture was kept on ice for 30 min before the cells were pelleted and washed with 40 ml ice-cold 0.1 M $CaCl_2$. Finally, competent cells were resuspended in 4 ml of ice cold 0.1 M $CaCl_2$ containing 10% glycerol for every 100 ml culture, aliquoted and frozen at -70°C.

Cells to be used for transformation by electroporation were inoculated from one colony into 5 ml of medium for culture overnight. Next morning 500 ml of medium was inoculated with the 5 ml overnight culture and the cells were grown for 3-4 hours until the turbidity of the culture (OD_{600}) reached 0.5-0.6. The culture was chilled on ice-water for 15 min and then centrifuged at 5,000 X g for 5 min at 4°C. The cell pellet was resuspended and washed with 500 ml of ice-water followed by 40 ml of ice-cold 10% glycerol in water. Finally the cells were resuspended in 500 µl of ice-cold water containing 10% glycerol and stored at -70°C.

II.4 Enzymes and reagents

Restriction enzymes were purchased from Boehringer Mannheim, GIBCO BRL Life Technology and New England Biolabs. Other enzymes for molecular biology (T4 DNA ligase, Klenow fragment, calf alkaline phosphatase, etc.) were supplied by Boehringer Mannheim. T4 polynucleotide kinase and M-MuLV reverse transcriptase were obtained from New England Biolabs. Taq DNA polymerase was from GIBCO BRL Life Technology Inc. All restriction endonuclease digestions and other reactions were performed according to the instructions provided by the manufacturers. The RNase inhibitor RNasin was purchased from Promega. The sequencing reagents and Sequenase for dideoxy sequencing were obtained from United States Biochemical.

All radioisotopically labeled compounds were produced by Amersham Inc., including [α-^{32}P]-dATP (3000 Ci/mmol), ^{125}I (4.0

GBq/ml), [γ-^{32}P]-ATP (3000 Ci/mmol), [α-^{32}P]-UTP (800 Ci/mmol) and [^{35}S]-methionine (800 Ci/mmol). Nitrocellulose for electrophoretic transfer of DNA and proteins was purchased from Schleicher and Schuell, Inc. Filters for colony hybridization and plaque lifts were supplied by Millipore.

Acrylamide, bis, ammonium persulfate, TEMED, SDS, electrophoresis grade agarose and low-melting-point agarose for gel electrophoresis were all obtained from Bio-Rad. Protein molecular weight markers were purchased from Pharmacia. Lambda *Hin*dIII DNA size markers were supplied by Boehringer Mannheim.

Universal and reverse primers for DNA sequencing and hexamer random primers for DNA probe labeling were supplied by Boehringer Mannheim. Specific primers for sequencing and oligonucleotides for DNA amplification by PCR were synthesized by the Department of Microbiology or Department of Biochemistry, University of Alberta. X-OMAT AR X-ray film was obtained from Eastman Kodak Co. All other reagents and chemicals were obtained from Sigma Chemical Co., Fisher Scientific Ltd., or where otherwise noted in the methods.

II.5 Isolation of genomic and plasmid DNA

Chlamydial chromosomal DNA was isolated from purified EBs as described (Wenman and Lovett, 1982). EBs from 30 culture flasks were suspended in a 30 ml of TES and pelleted at 35,000 X g for 20 min at 4°C and the pellet was resuspended in 4.5 ml solution containing 50 mM Tris-HCl (pH 8.0), 30 mM EDTA and 25% sucrose.

To this suspension, sarkosyl (30%) and proteinase K (10 mg/ml) were added to final concentrations of 0.8% and 0.2 mg/ml, respectively. The lysate was incubated at 56°C for 15 min followed by incubation at 37°C for 45 min. DNA was then extracted with phenol followed by chloroform. The total volume of the chromosomal DNA solution was adjusted to 6 ml with TE. 15.12 g of CsCl which was dissolved in 6 ml of TE was added slowly to the DNA mixture. The DNA was centrifuged at 100,000 X g for 16 hours at 16°C. The chromosomal DNA was collected and dialyzed against 3 changes of 2,000 ml of 1 X DNA buffer at 4°C to remove CsCl.

Plasmid DNA and replicative forms of bacteriophage M13 (RF) from *E. coli* were isolated by the alkaline lysis method of Birnboim and Doly (1979). A 100 ml overnight culture harboring a recombinant plasmid was pelleted by centrifugation at 5,000 X g for 10 min at 4°C. The cell pellet was resuspended in 5 ml of solution I containing 50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA. The suspension was then lysed with 10 ml solution II (0.2 N NaOH and 1% SDS) on ice for 10 min followed by neutralization with 7.5 ml ice-cold solution III containing 5 M potassium acetate pH 4.8. After 10 min on ice, the lysate was spun at 48,000 X g for 20 min at 4°C. The supernatant was transferred to a 30 ml Corex tube and 0.6 volume of isopropyl alcohol was added and mixed well. The mixture was centrifuged at 8,000 X g for 45 min at 22°C after standing for 15 min at room temperature. The pellet was washed with 70% ethanol and redissolved in 4 ml TE. 4 g of CsCl and 0.4 of ml ethidium bromide (10 mg/ml in H_2O) were added, followed by centrifugation at 100,000 X g for 16 hours at 15°C. Plasmid DNA was collected by puncturing with a 16 gauge (1 1/2 inch) needle into the tube directly beneath the lower band (closed circular plasmid DNA). Ethidium bromide was removed by extracting with 1-butanol saturated with water several times. The plasmid DNA was dialyzed in 2,000 ml of DNA buffer with three changes to remove CsCl.

For small volumes of culture, 1.5 ml overnight culture was pelleted and treated with solution I, II and III as described above or by the boiling method (Holmes and Quigley, 1981) in which the pellet was resuspended in STETL (8% sucrose, 0.5% Triton X-100, 50 mM Tris-HCl pH 8.0, 50 mM EDTA and 0.5 mg/ml lysozyme) and heated in boiling water for 30 seconds. The supernatant, after centrifugation, was extracted with phenol/chloroform and precipitated with ethanol or purified by use of a plasmid DNA purification kit provided by QIAGEN Inc. The plasmid DNA was dissolved in TE containing 0.1 mg/ml RNase A.

II.6 Gel electrophoresis of DNA and protein

DNA fragments in the range of 500 bp to 20,000 bp produced by digestion with appropriate restriction endonucleases were separated by electrophoresis at 5 volt/cm for 1-3 hours using a 0.8-1.0% agarose submarine gel in a horizontal container filled with 1 X TAE (40 mM Tris-acetate, 1 mM EDTA). Gels were visualized under ultraviolet light after staining with ethidium bromide. 4-6% polyacrylamide gel and 1 X TBE running buffer (45 mM Tris-borate and 1 mM EDTA) were used to resolve DNA fragments smaller than

500 bp by electrophoresis at 100 volt/per gel for 1-2 hours. DNA fragments excised from agarose gel were purified using a GeneClean kit (Bio 101), whereas DNA fragments recovered from polyacrylamide gel were eluted using a DNA elution buffer containing 0.5 M NH_4Ac, 10 mM $MgCl_2$ and 1 mM EDTA at 37°C overnight.

Protein samples were dissolved in 10-15 μl final sample buffer (6.25 mM Tris-HCl pH 6.8, 2.5% SDS, 12.5% glycerol, 0.01% bromophenol blue and 0.5% β-mercaptoethanol) and boiled for 3 min before loading, otherwise specified in the text, and resolved on SDS-PAGE (Laemmli, 1970). The stock solution of acrylamide : bis was 29% : 1%. All polyacrylamide gels were run with 0.75 mm spacers in a Bio-Rad mini-PROTEAN II vertical apparatus. The acrylamide concentration was 10-15% for the separating gel and 4.5% for the stacking gel. The quantity of protein in each sample was about 10-30 μg for each lane of gel, and standard protein low molecular weight markers from Pharmacia were included. Each protein gel was electrophoresed at 20 mA in 1 X running buffer for 2 hours and the resolved proteins were stained with Coomassie Brilliant Blue R-250 stain. Gels were destained in 35% methanol, 10% acetic acid. Any gel from which protein was to be eluted was cast with a wider comb and its two side edges were cut off for staining with Coomassie Brilliant Blue R-250. The stained gel edges were aligned with the unstained gel. A gel strip containing a protein of interest was cut out and placed in dialysis tubing filled with TE buffer. The protein was eluted by electrophoresis at 80 volts for 30-40 min in SDS running buffer. The eluted protein was concentrated in an Amicon centrifugal ultrafiltration tube at 4,000 rpm for 30-60 min.

II.7 Immobilization of DNA and proteins

The transfer of DNA to nitrocellulose has been described by Southern (Southern, 1975). After electrophoresis and staining with ethidium bromide, gels resolving DNA fragments were treated with 0.25 M HCl for 2 X 15 min followed by 0.5 M NaOH for 2 X 30 min. After the gels were neutralized with a solution containing 0.5 M Tris-HCl pH 7.5 and 3 M NaCl for 2 X 30 min, they were laid on a 3 MM Whatman paper bridge connected to a 10 X SSC reservoir. A piece of gel size nitrocellulose was placed on top of the gel with 2 layers of 3 MM Whatman paper and about a 10 cm stack of paper towels to draw the 10 X SSC upward through the gel. As the buffer flows upward, the DNA is transferred to the nitrocellulose. After 16-20 hours, the paper towels were removed, and the nitrocellulose was washed with 6 X SSC and baked at 80°C for 2 hours for subsequent hybridization.

The electrophoretic transfer of proteins to nitrocellulose has been described by Towbin et al (1979). After electrophoresis, a gel size piece of nitrocellulose, pre-wetted with Western transfer buffer, was laid on the gel. The gel and nitrocellulose were sandwiched with scouring pads and transferred in a Bio-Rad mini Trans-Blot filled with Western transfer buffer at 400 mA for 30 min. The nitrocellulose was stained with amido black for 1-2 min to visualize the proteins and molecular weight markers transferred from the gel to the nitrocellulose. The protein bands of interest, and the molecular weight markers, were marked with a pen and the nitrocellulose was prepared for reaction with specific antibodies.

Immobilization of DNA on nitrocellulose filters after colony or plaque lifting, in preparation for subsequent hybridization, was as follows (Grunstein and Wallis, 1979): a nitrocellulose filter was placed on the plate containing bacterial colonies or bacteriophage plaques and marked with pin pricks. The marked nitrocellulose was then carefully lifted with forceps after it was wet. Usually, bacteriophage-bearing plates were refrigerated for 10 min to ensure the integrity of the soft agar layer while lifting before the nitrocellulose is placed on the soft agar. After lifting, the nitrocellulose filters were laid on 2 sheets of 3MM Whatman paper soaked with 0.5 M NaOH for 10 min, neutralized with 1 M Tris-HCl pH 7.5 plus 1.5 M NaCl for 10 min twice, finally soaked in chloroform for 1 min and air dried for 15 min. The filters were baked at 80°C in a vacuum oven for 2 hours to ensure binding of DNA to the nitrocellulose for hybridization.

II.8 Labeling of DNA fragments

Random primer labeling (Feinberg and Vogelstein, 1983) was used to label specific DNA fragments for use as probes. 0.2-1.0 µg template DNA and 75 ng of random primer were boiled for 3 min and cooled on ice for 5 min. To the mixture of DNA, 20 µCi of $[\alpha-^{32}P]$-dATP, 2 units of Klenow fragment of *E. coli* DNA polymerase I and 4 µl of 5 X buffer containing 250 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM each dATP, dCTP, dTTP and 1 M HEPES were added in a total volume of 20 μl. The reaction was carried out at room temperature for at least 4 hours for maximal incorporation of radiolabel into the newly synthesized strand. A Sephadex G75 column (Pharmacia) was used to separate unincorporated [α-^{32}P]-dATP from labeled DNA fragments.

10 pmol of synthetic oligonucleotide primers were labeled with 50 μCi [γ-^{32}P]-ATP and 5 units of T4 polynucleotide kinase at 37°C for 30 min followed by adding 2 μl of 0.5 M EDTA to stop the reaction and heating at 68°C for 10 min to inactivate the enzyme. Unincorporated [γ-^{32}P]-ATP was separated from radioactively labeled oligos using a Sephadex G10 column (Pharmacia).

II.9 Iodination of protein A

Protein A (100 μg) was labeled with ^{125}I by the lactoperoxidase method (Morrison, 1980 in a reaction mixture containing 1 μl of ^{125}I, 20 μl of a 0.1 mM KI solution, 1 unit of lactoperoxidase in phosphate buffer (0.2 M, pH 7.0), 20 μl 40% H_2O_2, in a preincubation total volume of 135 μl. The reaction was incubated at room temperature for 7 min, and more lactoperoxidase (1 unit) and H_2O_2 (20 μl of 40%) were added. The incubation was continued for an additional 10 min and then NaN_3 (20 μl of a 25% solution), KI (25 μl of a 0.1 mg/ml solution) and BSA (25 μl of a 1% solution in phosphate buffer) were added. This whole reaction mixture was applied to a column of Sephadex G25 over an ion exchange resin (AG-1X8 acetate form) and eluted with 1% BSA. Fractions of 1 ml were collected and the reaction efficiency determined by precipitation with trichloroacetic acid (TCA) and monitoring in a LKB RackGamma II gamma counter.

II.10 DNA hybridization and protein blotting

Baked DNA blots for DNA hybridization from the Southern transfer, colony or plaque lifting were pre-wet in 6 X SSC and transferred to prehybridization solution made of deionized formamide (5.0 ml), 20 x SSC (2.5 ml), 1 M Tris-HCl pH 7.5 (0.5 ml), 50 x Denhardt's (1.0 ml; 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA), and 0.4 ml of 2.5 mg/ml salmon sperm DNA (boiled for 5 min and placed on ice immediately) for blocking in a total volume of 10 ml at 37°C for 1-3 hours. Formamide was deionized with a mixed-bed ion exchange resin (1:1 of AG50W-X8: AG1-X8) by stirring for 30 min at room temperature and filtering with Whatman #1 filter. Hybridization solution was the prehybridization solution plus a volume of probe boiled for 5 min, representing 1 x 10^6 counts per minute (c.p.m.). Hybridization reactions were carried out at 37°C overnight with agitation and hybridized DNA blots were washed with 2 X SSC, 0.2% SDS in a 65°C water bath for 10 min twice with agitation followed with 0.2 X SSC in 65°C water bath for 10 min twice. The blots were air-dried for 15 min and subjected to autoradiography.

All autoradiography was performed using Kodak X-OMAT AR X-ray film or Fuji medical X-ray film with or without Dupont Cronex Lightning-Plus intensifying screens. Exposures were done at -76°C for varying times. Films were developed in Kodak automatic X-ray film processors.

The amido black-stained nitrocellulose, containing proteins transferred from polyacrylamide gel, was washed with distilled water several times to reduce the stain background and placed into blocking solution (5% fat-free milk or 2% BSA in 1 X PBS) for 1-2 hours at room temperature with mild agitation. Antibodies diluted in 5% milk or 1% BSA in PBS at a ratio of 1:100 to 1:1000 were incubated with the protein blot from 2 hours to overnight at room temperature. The blot was washed with PBS, 0.1% Tween 20 for 5 min three times and then reacted with second antibodies, [^{125}I]-labeled Protein A, or non-radioactive labeled goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) for 1 hour at room temperature followed by three washes with PBS, 0.1% Tween 20. The blots using ^{125}I-labeled Protein A as a second antibody were exposed to X-ray film directly, while non-radioactive labeled blots were treated with horseradish peroxidase substrate 4-chloro-1-naphthol. The color development was stopped by washing the blots with a few changes of water. The colored blots were photocopied and kept in aluminum foil.

II.11 Genomic library and Cloning of restriction fragments

Genomic DNA isolated from *C. trachomatis* serovar L2 and vector DNA of the pUC series or pBluescript SK+ were digested with appropriate restriction endonucleases for 2 hours, extracted with phenol/chloroform and precipitated with ethanol. Vector DNA that had been cut with a single restriction enzyme was dephosphorylated to reduce self-ligation. Wherever possible, forced directional ligation were performed. These had the two ends (of both vector and insert) cut with different restriction endonucleases. A vector to insert ratio of 1:3 was used. Ligation reactions were carried out in a total volume of 10 µl containing 0.01 Weiss unit of ligase for DNA fragments with overhanging ends and 0.5 Weiss unit for blunt ends at 16°C overnight or at room temperature for 4 hours. The ligation mixtures were diluted with TE 5-fold and 1 µl (~10 ng DNA) of diluted ligation mix was used for each transformation of 100 µl competent cells. The competent cells were mixed with ligated DNA on ice for 45 min followed by heat shocking at 42°C for 60-90 seconds. After remaining on ice for five min, the cells were added to 1 ml of LB medium and grown at 37°C for 1 hour to allow expression of β-lactamase (ampicillin-resistance). The recombinants were identified by the absence of hydrolysis of X-gal coated on the agar in plates in the presence of IPTG. Ligation of foreign DNA into the polylinker region of the pUC-like vectors interrupts the *lacZ* gene. Therefore cells bearing these plasmids cannot hydrolyze the X-gal on the plate and these colonies remain white. Cells carrying non-recombinant plasmids can hydrolyze the X-gal on the plate and therefore these colonies turn blue in color. The further identification of DNA fragments from colonies or plaques was accomplished by DNA-DNA hybridization. Some cloned restriction fragments were subcloned into bacteriophage M13 mp18 or mp19 for the purpose of single-stranded sequencing (Messing, 1983) or further cut with appropriate enzymes to generate a restriction map of the fragments. Small DNA fragments were then subcloned for double-stranded sequencing.

II.12 DNA sequencing

All DNA sequencing was performed using the dideoxy chain termination method (Sanger et al. 1977) with [α-^{32}P]-dATP and the sequenase 2.0 sequencing kit (United States Biochemical Corp., Cleveland, Ohio). 200 ng of single-stranded DNA templates, together with 0.2 pmol of primers and 5 X sequenase buffer containing 200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$ and 250 mM NaCl in a volume of 10 µl, was incubated at 70°C for 2 min and cooled slowly to room temperature (over 30 min) to anneal the primers to the templates. Sequencing labeling reactions were accomplished by adding a reaction mix made of 1 µl of 0.1 M DTT, 1.71 µl H_2O, 2 µl TE pH 7.4, 0.43 µl labeling mix (7.5 µM of dGTP, dCTP, dTTP each), 1 µl [α-^{32}P]-dATP (3,000 µCi/ml) and 0.28 µl Sequenase to primer annealed template DNA. Labeling reactions usually required 2-5 min at room temperature and the reaction mixture was divided into aliquots to which 3.5 µl of termination nucleotide mixtures (80 µM four dNTPs with 8 µM one of four ddNTPs) was added for 5 min at 37°C to stop the DNA chain elongation by incorporation of one of the ddNTPs into newly-synthesized DNA molecules. The entire sequencing reaction was terminated with addition of 4 µl of sequencing dye (95% formamide v/v, 20 mM EDTA, 0.05% bromophenol and 0.05% xylene cyanol FF). For double stranded DNA sequencing (Wang, 1988), 2.5 µg DNA was denatured with 0.2 N NaOH for 10 min at 37°C followed by neutralization with 1/10 volume of 3 M NaAOc pH 5.2 and precipitation with ethanol. 2.5 pmol of primer was used for each double stranded DNA sequencing reaction and the primer was annealed for 15 min at 37°C in a volume of 10 µl. Universal primer and reverse primer for either sequencing reaction or PCR were 17 mer with the sequence 5'-GTA AAA CGA CGG CCA GT-3' and 5'-CAG GAA ACA GCT ATG AC-3', respectively. All cloned DNA restriction fragments and PCR generated DNA fragments were sequenced on both strands.

The stock solution of acrylamide:bis for DNA sequencing was 38%:2%. Gels of 4% and 6% were used for long gels (19 cm wide x 85 cm tall, 0.25 mm thick) and short gels (34 cm wide x 40 cm tall, 0.25 mm thick) respectively. Acrylamide monomer was dissolved in a solution of 8.3 M urea and 0.1 M TEB and cast between glass plates. The sequencing running buffer was 60 mM TEB and gels were pre-run for 1 hour at 75 watts for long gels and 50 watts for short gels. Gels were loaded with 1.5-2 µl of sequencing sample in each lane and run under the same conditions as the pre-run. A 6 mm thick gel-size aluminum plate was used to spread the heat generated by electrophoresis.

II.13 Computer analysis of DNA and amino acid sequences

DNA Strider 1.2 software for the Macintosh computer (C. Marck, Service de Biochimie, Centres d'Etudes Nucleaires de Saclay, France) (Marck, 1988) was used for DNA or amino acid sequence compilations, 6 phases of open reading frame analyses (ORF), restriction and graphic maps of DNA sequences, protein translation analyses, hydropathy map of proteins. Amino acid composition, codon usage, molecular weight and pI of proteins and protein secondary structures were analyzed on MacVector 3.5 (IBI-A Kodak Company, New Haven, CT). Intelligenetics software (Intelligenetics Inc., Mountain View, CA, USA) in a SUN computer with UNIX system and GCG (Genetics Computer Groups Inc., Madison, Wisconsin) programs were used for homology searches of DNA and peptide sequences, sequence comparison and alignments, sequence from databanks, terminator sequence analysis and sequence displays.

II.14 Nucleotide sequence accession numbers

The nucleotide sequence data reported in this thesis have been submitted to GenBank and have been assigned accession numbers M80325 for the *spc* r-protein gene operon, L25077 for the *secY* gene and L33834 for the α r-protein gene operon.

II.15 Plasmid-directed synthesis of proteins

Some DNA fragments containing coding regions were subcloned into plasmids of the pUC series under the control of the *lacZ* promoter for protein expression or into expression plasmid pT7-7 (ϕ10 promoter) in frame or into expression vector pGEX-3X in frame to generate glutathione-S-transferase (GST) fusion proteins. Medium inoculated with overnight culture at a ratio of 1:50-100 was grown for 2-3 hours (OD_{600}: ~0.3-0.4) with selective conditions. 0.4-4 mM IPTG was used to induce specific protein synthesis from the expression vector at various times from 30 min to 5 hours. 0.1 mM IPTG was used for insoluble protein products. The cells were then harvested, pelleted, resuspended in final sample buffer and subjected to gel electrophoresis or further protein purification.

To label specific plasmid-directed proteins synthesized by T7 RNAP, *E. coli* strain BL21 was grown in minimal media and 0.2 mg/ml of rifampicin was added to inhibit the protein synthesis of host cells 30 min after the addition of IPTG (to induce the T7 RNAP gene expression). Incubation was continued for 30 min followed by labeling the protein synthesized by T7 RNAP for 5 min with 10 µCi/ml of [^{35}S]-methionine (Amersham). After protein electrophoresis, the gels were dried and exposed to X-ray film over night.

II.16 Generation of polyclonal antibodies

All polyclonal antibodies were generated based on the procedure described by Harlow and Lane (1988). Rabbits used for polyclonal antibody generation were New Zealand white, female and 10-12 weeks old. Proteins purified from overexpressed recombinant clones and eluted from polyacrylamide gels were concentrated to 0.5-1.0 mg/ml using an Amicon ultrafiltration tube. 1 ml of protein solution was mixed thoroughly with the same volume of Freund's complete adjuvant and used for the first intramuscular injection. The rabbits were boosted with a mixture of the protein and Freund's incomplete adjuvant 4 weeks after the first immunization. The antibodies were tested by Western Blot 2 weeks after the boost.

II.17 *In vitro* amplification of DNA by PCR

In vitro amplification of DNA by PCR was performed as described (Mullis and Faloona, 1987). Reactions were carried out in a total volume of 100 µl containing 100 pmol of each primer, 200 µM of each dNTP, 0.1 ng of cloned template DNA (or 0.1 µg of chromosomal DNA) and 2 units of Taq DNA polymerase. 10 X reaction buffer was composed of 500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$ and 0.1% gelatin. The template DNA was denatured at 94°C for 3 min first and the reaction mixture was cycled with three different temperature segments: denaturing (94°C), annealing (depending on Tm of oligos; determined by adding 4°C for each G or C and 2°C for each A or T) and DNA extension synthesis (72°C). 25-30 cycles for each reaction was followed by 4°C. All PCR reactions were performed on a GeneAmp PCR System 9600 (Perkin Elmer).

II.18 RNA isolation

HeLa cells infected with Chlamydial EBs at various times were harvested and lysed with a buffer of 0.1 M Tris-HCl pH 7.5, 0.2% SDS, 50 mM NaCl and 10 mM EDTA. The lysate was extracted with hot acidic phenol/chloroform three times at 65°C (phenol saturated with 50 mM NaAOc pH 5.0 and heated to 65°C) followed by extraction with ether three times at room temperature. RNA was precipitated by addition of two volumes of 100% ethanol, followed by storage at -70°C for 30 min, collected by centrifugation, and used directly for further analysis.

II.19 Isolation of *E. coli* RNAP holoenzyme

Whole RNAP from *E. coli* XL-1 cells harboring either the SK+ or recombinant pCTA1 expressing the chlamydial α subunit was isolated according to the method of Burgess and Jendrisak (1975) with some modifications by Kumar and Chatterjee (1988). All purification steps were carried out at 0-4°C. Total cellular extracts of the cells containing recombinant or control DNA were precipitated by addition of polymin P followed by sequential precipitation with NaCl and ammonium sulfate. Following ammonium sulfate precipitation the protein pellet was dissolved in TGED buffer (10 mM Tris-HCl pH 7.9, 5% glycerol, 0.1 mM EDTA, 0.1 mM DTT) containing 0.5 M sodium chloride and applied to a Bio-Gel-A-1.5m column (100 to 200 mesh). RNAP-rich fractions eluted from the column were pooled and concentrated once again by ammonium sulfate precipitation. The precipitate was dissolved in TGED containing 0.3 M sodium chloride and dialyzed against the same buffer overnight. The dialyzed material was applied to a heparin-sepharose CL-6B column. The column was washed with the same buffer and then eluted with TGED containing 0.6 M NaCl. The resultant protein solution was concentrated by centrifugation through Microcon-30 (Amicon Inc., MA) and stored at -20°C.

II. 20 Time course of α subunit protein expression

HeLa monolayers were grown in 24-well tissue culture plates for 24-36 hours in MEM supplemented with 10% heat treated fetal calf serum. Each adherent monolayer was inoculated with 2 X 10^5

inclusion forming units of purified L_2 EBs in a final volume of 200 µl of HBSS. The plates were incubated at room temperature for 2 hours with rocking. At the end of the incubation period, unbound EBs were aspirated and the cells were provided with fresh medium containing 1 µg/ml cycloheximide and 25 µg/ml gentamicin. The EB-infected cells were incubated at 37°C for 48 hours. At desired time points cell monolayers were washed twice with cold HBSS, dissolved in 200 µl final sample buffer and removed from microtiter wells. Samples were lysed by boiling for 3-5 min and then analyzed on 12.5% SDS-PAGE. The proteins were transferred to nitrocellulose and probed with polyclonal antibodies.

II.21 Isolation of chlamydial host-free extracts containing RNAP

Isolation of chlamydial host-free extracts containing RNAP from RBs harvested at 18-20 hours post infection was based on a procedure published by Mathews et al (1993). The RBs isolated from six or two large flasks (for high or low concentration of chlamydial RNAP) of HeLa cells (175 cm^2) infected with *C. trachomatis* L2 were purified by renografin density gradient centrifugation. The RBs were resuspended in 400 µl of TDG buffer consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 3 mM DTT, 10% glycerol, 10 mM $MgCl_2$, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml of pepstatin A. The suspension was lysed by the addition of 8 µl of 10% Nonidet P-40 followed by centrifugation at 10,000 X g for 10 min at 4°C. The pellet was washed with 400 µl of TDG and the centrifugation was repeated to yield supernatant 2 and pellet 2.

Pellet 2 was resuspended in 20 µl of TDG containing 2 M KCl to elute RNAP from DNA. After 10 min, a further 20 µl of TDG was added and mixture was centrifuged at 10,000 X g for 10 min at 4°C to produce the "salt supernatant" and pellet 3. Pellet 3 was washed with 40 µl of TDG buffer and centrifugation was repeated. The final supernatant was mixed with "salt supernatant" to become salt supernatant 2 (SS2) containing *Chlamydia* host-free RNAP. The SS2 was used directly for *in vitro* RNA synthesis reactions.

II.22 Purification of *Chlamydia* α subunit and chimeric *E. coli* RNAP

The gene encoding chlamydial RNAP α subunit was subcloned into the expression vector pGEX-3X using a PCR amplified DNA fragment. The recombinant plasmids were electroporated into *E. coli* host BNN103 in a 0.1 cm gap electrode cuvette at 1.8 kV and 25 µF on a Bio-Rad Gene Pulser. The cells were suspended in 1 ml of SOC immediately after electroporation discharge and grown at 37°C for 1 hour with agitation at 200 rpm. To intended increase the solubility of GST-α fusion protein, induction of a 1 liter culture was carried out by addition of IPTG to 0.1 mM at 28°C growth temperature and an OD_{600} 0.5-0.6. The cells were harvested 1 hour after IPTG induction and briefly sonicated. The suspension was pelleted and 1 ml of 50% glutathione Sepharose slurry was added to the supernatant. The fusion-protein-bound-glutathione Sepharose beads were washed with PBS three times (10 X bed volume) to remove any unbound proteins after incubation at 4°C for 3 hours with gentle agitation. The fusion protein-bound matrix was then washed with 10 volumes of factor Xa cleavage buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl and 1 mM $CaCl_2$). 50 μg of factor Xa and 1 ml of factor Xa cleavage buffer were added to the washed fusion protein-bound matrix. The factor Xa cleavage reaction was carried out at 4°C for 16 hours and analyzed by electrophoresing 2 μl of mixture on SDS-PAGE. The reaction suspension was pelleted at 500 X g for 5 min and the supernatant containing free chlamydial α subunits was either subjected to further purification by glycerol continuous gradient centrifugation (see next) or dialyzed in a storage buffer (Borukhov and Goldfarb, 1993) consisting of 40 mM Tris-HCl pH 7.9, 0.2 M KCl 50% glycerol, 1 mM EDTA and 1 mM DTT.

4 ml of the 15% to 30% glycerol gradient (Hayward et al. 1991) containing 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and 0.2 M NaCl was used to separate overexpressed free chlamydial α protein from small amount of hybrid *E. coli* RNAP co-purified with incorporated chlamydial α subunit through a glutathione affinity column. The protein mixture was centrifuged in a Beckman SW55 Ti rotor at 40,000 rpm (155,000 X g) and 4°C for 17 hours. 12 fractions of 0.4 ml were collected and 20 μl samples from each fraction were tested by SDS-PAGE. Fractions 2, 3 and 4 containing hybrid *E. coli* RNAP and fractions 9, 10 and 11 containing pure chlamydial α subunit were kept at -20°C.

II.23 *In vitro* transcription assay

In vitro transcription assays (Mathews et al. 1993) were carried out in a total volume of 100 μl. 10 μl of *Chlamydia* extract

SS2 containing host-free RNAP was added to the reaction mixture. The components of this mixture were 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 50 mM KCl, 100 µg/ml PMSF, 10 µg/ml of pepstatin, 100 units of RNasin, 330 µM each of ATP, CTP and GTP, 1 µM UTP, 25 µCi of [α-^{32}P]-UTP and 2.5 µg of template DNA. The total reaction mixture was incubated at 37°C for 20 min and the reaction was terminated by adding 100 µl of termination buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2% SDS and 2 µg of *E. coli* tRNA. The terminated reaction mixture was extracted with hot acidic phenol (65°C) saturated with 50 mM NaAOc pH 5.2 three times to remove the template DNA followed by three ether extractions. RNA was precipitated with ethanol and redissolveded in 100 µl of 0.1% diethylpyrocarbonate treated water and heated to 100°C before use as a probe.

II.24 DNA mobility shift assay

The DNA mobility shift assay was based on the method of Musgrave et al (1991). ^{32}P radioactively labeled 680 bp DNA fragments containing the chlamydial MOMP promoter region were mixed with various concentrations of purified chlamydial α subunit protein in molecule ratios ranging from 1:0 to 1:10. The DNA:protein binding reaction was carried out at room temperature for 15 min in a buffer consisting of 60 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.01 mM EDTA, 0.01 mM DTT, 15% glycerol and 1 mg/ml BSA. A 550-bp DNA fragment containing a partial MOMP gene was used as a control under the same reaction conditions. The DNA:protein complexes were resolved by electrophoresis on 4% polyacrylamide gels in 1X TAE buffer at 50 volts for 2 hours. Labeled DNA was detected by autoradiography with or without Dupont Cronex Lightning-Plus intensifying screens at -70°C for varying times.

II.25 Primer extension assay

The primer extension assay was performed as described by McKnight and Kingsbury (1982). The reaction was carried out in a volume of 20 µl containing 100 µg of total RNA isolated from *Chlamydia* infected HeLa cells, 1 µM of ^{32}P labeled primer, 1 mM each dNTP, 50 µg/ml actinomycin D, 1 µl of 10 X reverse transcriptase (RT) buffer and 40 units of reverse transcriptase. The RT mixture was incubated at 42°C for 90 min and the reaction was terminated by addition of 2 µl of 0.5 M EDTA, followed by heating at 90°C for 1 min. The reaction mixture was incubated with 1 µg of RNase at 37°C for 30 min followed by phenol/chloroform extraction and ethanol precipitation in the presence of 2 µg tRNA. The precipitated nucleic acids were collected by centrifugation and dissolved in 10 µl of sequencing buffer.

II.26 Preparation of genomic DNA agarose blocks

For CHEF (Contour-clamped Homogeneous Electric-Field) gel electrophoresis (Chu et al. 1986), whole EBs were lysed in Seaplaque agarose blocks. Usually 1 ml of EBs (5 mg) were washed twice with PBS, resuspended in 1 ml of PBS, mixed with an equal volume of warm 1.5% Seaplaque agarose in 0.25 M EDTA and finally distributed into molds. The resulting agarose blocks were incubated for 48 hours at 50°C in ES (0.2 M EDTA, 1% sarkosyl) buffer containing 2 mg/ml proteinase K, then washed for 30 min twice with TE containing 1 mM PMSF, and three times in storage TE (10 mM Tris pH 8.0, 100 mM EDTA pH 8.0).

II.27 Pulsed-Field Gel Electrophoresis

Agarose blocks (2 by 2 by 5 mm) containing 1 μg of genomic DNA of *C. trachomatis* were immersed in TE for 30 min on ice, transferred into 100 μl volumes of appropriate restriction endonuclease buffers for 30 min and then digested with restriction enzymes *Not*I (GC|GGCCGC) (Boehringer Mannheim Canada Ltd.), *Sgr*AI (C(A/G)|CCGG(T/G)G) (Boehringer Mannheim) and *Sse*8387I (CCTGCA|GG) (Takara Biochemical Inc. Berkeley, Calif.) overnight at 37°C. DNA inserts were then loaded into wells in a 1% agarose gel in 0.5 M TBE. The gel was electrophoresed at 10°C, 8 V/cm in a CHEF II apparatus (LKB 2015, Pulsaphor™ system). For 50 kb to 500 kb DNA fragment resolution, the pulse switch time was 5 seconds for 16 hours, followed by 30 seconds for 8 hours, while for sizes between 8 kb and 250 kb, the pulse was 3 seconds and 20 seconds respectively. A ladder of lambda DNA concatamers (BioLabs, New England) was used for DNA size markers. The gel was stained with 0.5 μg/ml ethidium bromide for 30 min.

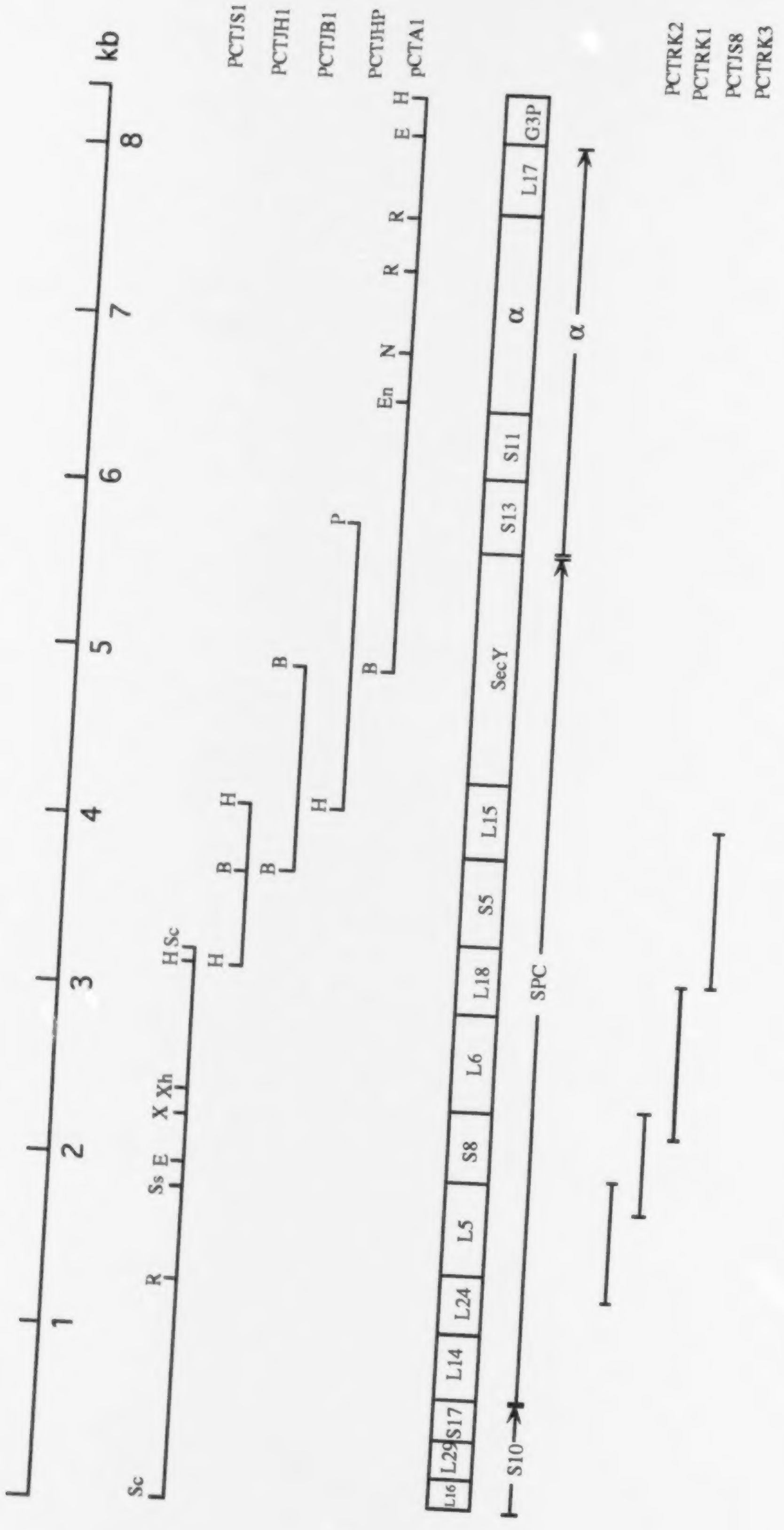
III. Results

III.1 Cloning and sequence analysis of the *C. trachomatis S10-spc-α* r-protein gene cluster

III.1.1 Cloning of the *C. trachomatis S10-spc-α* operon. A 3.2-kb *Sac*I fragment designated pCTJS1 that encoded the r-protein CtrL6e (Gray et al. 1991) has been isolated from a *C. trachomatis* gene library and reported from our laboratory. The DNA sequence of an internal 1.2-kb *Xba*I-*Hin*dIII fragment designated pCTJS8 revealed the presence of genes for proteins homologous to EcoS8 and EcoL18 flanking the CtrL6e. This is reminiscent of the *spc* operon genes in *E. coli*. Consequently, the sequence of the whole 3.2-kb *Sac*I fragment of pCTJS1 was determined. DNA sequence analysis of the *Hin*dIII-*Sac*I fragment adjacent to the previously sequenced *Hin*dIII site (from plasmid pCTJS8) revealed that this region corresponded to the carboxyl terminus of CtrL18e. In order to extend the sequence to cover the entire *spc* region, overlapping DNA fragments were generated through successive subcloning and detected by hybridization using appropriate DNA fragments as probes. A restriction map, including the sequencing strategy and gene organization of this *C. trachomatis* r-protein gene cluster, is shown in Fig. 1.

By using DNA walking and subcloning strategies an overlapping 1.7 kb *Hind*III-*Pst*I fragment (PCTJHP) was identified and cloned using a 840 bp *Hin*dIII-*Bam*HI fragment (nt 4080-4920) as a probe (Fig. 1). Sequence analysis of this 1,742 bp *Hind*III-*Pst*I

FIG. 1. Physical map, sequencing scheme and subcloning strategy of a 8,350-bp *C. trachomatis* DNA segment containing the *S10-spc-α* operon gene cluster. Restriction sites are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; En, *Eco*NI; H, *Hin*dIII; N, *Nsi*I; P, *Pst*I; R, *Rsa*I; Sc, *Sac*I; Ss, *Ssp*I; X, *Xba*I; Xh, *Xho*I. Coding regions are boxed and labeled with the gene product designations. Arrows indicate the direction of transcription. Regions corresponding to the *S10*, *spc* and *α* operons are labeled on the bottom. The clones named at the far right represent recombinant plasmids used for either expression or subcloning and sequencing studies.


kb
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PCTJS1
PCTJH1
PCTJB1
PCTJHP
pCTA1
L16
L29
S17
L14
L24
L5
S8
L6
L18
S5
L15
SecY
S13
S11
α
L17
G3P
S10
SPC
α
PCTRK2
PCTRK1
PCTJS8
PCTRK3

fragment showed that it consists of three ORFs, distal L15, SecY and proximal S13, the first gene of the α operon (Cerretti et al. 1983).

I took advantage of the fact that in *E. coli*, the α operon is present directly downstream of the *spc* operon. I hypothesized that the order of these two operons might be conserved in *Chlamydia*. In order to extend the sequence to cover the entire α region, DNA walking and subcloning strategies were employed. Using a 906-bp *Bam*HI-*Pst*I fragment (nt 4920-5814) of *secY* as a probe, an overlapping 3.4-kb *Bam*HI-*Hin*dIII (pCTA1) (Fig. 1) fragment of *C. trachomatis* serovar L_2 DNA was cloned. This contained the genes for the α core subunit of RNAP and adjacent genes for the S13, S11 and L17 r-proteins. Further downstream from the L17 stop codon is another ORF. The predicted amino acid sequence of this ORF is similar to that of the *E. coli* glyceraldehyde-3-phosphate dehydrogenase, a key enzyme of the glycolytic pathway.

III.1.2 Sequence analysis and gene organization. The DNA sequence as determined from analysis of both strands of the 8,350-bp region is shown in Fig. 2, together with the predicted amino acid sequences of the encoded proteins. The protein-encoding regions were identified by comparison with *E. coli* r-protein gene sequences by using the FASTP (Lipman and Pearson, 1985) and FASTA programs in the GCG collection. As shown, all putative r-protein genes in this cluster are initiated with ATG codons except the gene for CtrS11 which has a TTG start codon. The ORFs are terminated with either TAG or TAA codons except *secY* gene which

FIG. 2. Complete nucleotide sequence of the *C. trachomatis S10-spc-α* operon region. The predicted amino acid sequence for each gene is given in single letter code under the DNA sequence, and the ORF borders are indicated below the amino acid sequence. The asterisks represent stop codons. The numbers on the right of each row refer to nucleotide positions. The promoter sequences for the *spc* and *α* operons, some Shine-Dalgarno sequences and some restriction endonuclease sites are underlined.

```
Sac I
GAG CTC CTG ATC ACT GGG TAG CTG TTG TCC GTC CCG GAC GTA TTT TAT TCG AAG TGG CAA 60
  A   P   D   H   W   V   A   V   V   R   P   G   R   I   L   F   E   V   A   N
  Ctr L16e

ACG TTT CGA AAG AAG ATG CTC AGG ATG CTT TGA GAA GAG CTG CTG CAA AGT TAG GAA TTA 120
  V   S   K   E   D   A   Q   D   A   L   R   R   A   A   A   K   L   G   I   R

GAA CAC GAT TTG TTA AGC GTG TGG AAA GGG TAT AGT ATG GGA GCA AAA AAG AAT TTA TTA 180
  T   R   F   V   K   R   V   E   R   V   *     M   G   A   K   K   N   L   L
                               end Ctr L16e    start Ctr L29e

GCG GAG CTT AGA GAG AAG AGT TCT GAA GAG TTG GAT GAG TTT ATT CGT GAT AAT AAA AAA 240
A   E   L   R   E   K   S   S   E   E   L   D   E   F   I   R   D   N   K   K

GCT CTC TTC GCT TTG CGT GCG GAA GCT GCT TTA CAG AAT AAA GTT GTG AAA ACT CAT CAG 300
A   L   F   A   L   R   A   E   A   A   L   Q   N   K   V   V   K   T   H   Q

TTT TCT CTG TAT AAG AAA AGC ATT GCT CGT GCT CTA ATA ATA AAA CAA GAA AAA AAG GGT 360
F   S   L   Y   K   K   S   I   A   R   A   L   I   I   K   Q   E   K   K   G

AGA GTC CAT GGC TAG TGA TGT GAG AGG CCG TAG AAA GAC CAA AAT TGG TGT AGT AGT CTC 420
R   V   H   G   *   *  end Ctr L29e
         M   A   S   D   V   R   G   R   R   K   T   K   I   G   V   V   V   S
         start Ctr S17e
                        -35                             -10          +1
ATC AAA AAT GGA AAA AAC TGT TGT TGT TCG AGT CGA AAG GGT ATA CTC GCA CCC TCA ATA 480
 S   K   M   E   K   T   V   V   V   R   V   E   R   V   Y   S   H   P   Q   Y

TGC TAA GGT GGT TAG GGA TTC TAG CAA GTA TTA TGC GCA TAA TGA GTT GGA TGT GAA AGA 540
 A   K   V   V   R   D   S   S   K   Y   Y   A   H   N   E   L   D   V   K   E

AGG TGA TAC TGT TCG AAT CCA AGA GAC GCG TCC TTT GTC TAA AAC GAA GAG ATG GCG GGT 600
 G   D   T   V   R   I   Q   E   T   R   P   L   S   K   T   K   R   W   R   V

TGT CGG ACG TGT AAA TTA GTA GTG GTT TAG CAA TTA TGA TCC AGC AAG AAA GTC AGT TAA 660
 V   G   R   V   N   *   *  end Ctr S17e      M   I   Q   Q   E   S   Q   L   K
                                              start Ctr L14e

AAG TTG CCG ATA ATA CAG GGG CTA AGA AAG TTA AGT GTT TCA AGG TTC TAG GCG GAT CTC 720
  V   A   D   N   T   G   A   K   K   V   K   C   F   K   V   L   G   G   S   R

GTC GAC GTT ATG CAA CGG TCG GTG ATG TGA TTG TAT GCT CTG TAA GAG ATA TTG AGC CTG 780
  R   R   Y   A   T   V   G   D   V   I   V   C   S   V   R   D   I   E   P   D

ATA GTT CCG TAA AGA AGG GGG ATG TTG TTA AGG CTG TAA TCG TAC GGA CTC GAA ACG ATA 840
  S   S   V   K   K   G   D   V   V   K   A   V   I   V   R   T   R   N   D   I

TCC ATC GTA AAG ATG GTT CTA CAC TAA GAT TCG ATA CGA ATA GTT GTG TAA TCA TCG ATG 900
  H   R   K   D   G   S   T   L   R   F   D   T   N   S   C   V   I   I   D   D

ATA AAG GCA ATC CTA AAG GAA CTA GAA TTT TTG GGC CTG TAG CAA GGG AGA TTC GAG ACA 960
  K   G   N   P   K   G   T   R   I   F   G   P   V   A   R   E   I   R   D   R

GAG GCT TTG TTA AGA TTA GCT CTT TGG CTC CCG AGG TGA TTT AAA GGT AAG ATA GTA TGA 1020
  G   F   V   K   I   S   S   L   A   P   E   V   I   *  end Ctr L14e     M   K
                                                                 start Ctr L24e

AGA GAC GTA GTG TTT GTG TCG GTG ACA CTG TTT ATG TGC TTG CTG GAA ACG ACA AAG GTA 1080
  R   R   S   V   C   V   G   D   T   V   Y   V   L   A   G   N   D   K   G   K

AGC AAG GGA AAG TTT TAC GTT GTT TGA AGG ATA AGG TTG TTG TTG AAG GAA TCA ATG TCC 1140
  Q   G   K   V   L   R   C   L   K   D   K   V   V   V   E   G   I   N   V   R
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GAG TAA AAA ATA TTA AAC GCT CTC AAG AGA ATC CTA AAG GGA AGC GCA TTA ATA TTG AGG 1200
  V   K   N   I   K   R   S   Q   E   N   P   K   G   K   R   I   N   I   E   A
                          Rsa I
CTC CTC TCC ATA TTT CTA ACG TAC GTT TAA GTA TCG ATA ATC AGC CTG CTA GAC TGT TTG 1260
  P   L   H   I   S   N   V   R   L   S   I   D   N   Q   P   A   R   L   F   V

TCA AAG TTA CAG AGA AAG GAC GAG AGC TTT GGA ATA AGC ATT CCG ATG GAA GTT CTT CAT 1320
  K   V   T   E   K   G   R   E   L   W   N   K   H   S   D   G   S   S   S   L

TAT ACC GAT TGG TAA GAG AGA GAA AGG GTT AAT ATG AGC AGG TTA AAA AAA CTA TAT ACT 1380
  Y   R   L   V   R   E   R   K   G   *     M   S   R   L   K   K   L   Y   T
                           end Ctr L24e     start Ctr L5

GAA GAG ATA AGA AAG ACT CTT CAA GAT AAG TTT CAG TAT GAA AAT GTA ATG CAA ATC CCT 1440
E   E   I   R   K   T   L   Q   D   K   F   Q   Y   E   N   V   M   Q   I   P

GTT CTT AAG AAG ATC GTA ATA AGC ATG GGG CTT GCA GAG GCT GCA AAG GAT AAA AAC CTT 1500
V   L   K   K   I   V   I   S   M   G   L   A   E   A   A   K   D   K   N   L

TTC CAG GCT CAT TTA GAG GAA TTG GCG GTT ATC TCT GGT CAA AAA CCT TTG GTA ACA AGA 1560
F   Q   A   H   L   E   E   L   A   V   I   S   G   Q   K   P   L   V   T   R

GCT AAA AAC TCT ATC GCA GGC TTC AAG TTA CGA GAG GGT CAG GGC ATC GGA GCA AAA GTC 1620
A   K   N   S   I   A   G   F   K   L   R   E   G   Q   G   I   G   A   K   V

ACT CTA CGT GGA ATC CGT ATG TAT GAC TTT ATG GAC CGT TTT TGC AAT ATT GTC TCC CCA 1680
T   L   R   G   I   R   M   Y   D   F   M   D   R   F   C   N   I   V   S   P

AGA ATT CGA GAC TTT AGA GGA TTC TCT TGT AAA GGA GAT GGA CGA GGA TGT TAT TCC CTT 1740
R   I   R   D   F   R   G   F   S   C   K   G   D   G   R   G   C   Y   S   L

GGT TTA GAT GAT CAG CAA ATC TTT CCT GAA GTT GAT TTA GAT GCT GTT AAA CGA TCT CAG 1800
G   L   D   D   Q   Q   I   F   P   E   V   D   L   D   A   V   K   R   S   Q
        Ssp I
GGA ATG AAT ATT ACT TGG GTA ACT ACA GCA CAA ACC GAT GCG GAG TGC CTT ACC TTG TTA 1860
G   M   N   I   T   W   V   T   T   A   Q   T   D   A   E   C   L   T   L   L

GAG TGT ATG GGC TTG CGT TTC AAG AAG GCT CAA TAA GGG AGA TGT AGG TCG GTA TGG GAA 1920
E   C   M   G   L   R   F   K   K   A   Q   *  end Ctr L5e            M   G   M
                                                                  start Ctr S8e

TGA CGA GTG ATT CAA TCG CAA ATT TAT TGA CAC GGA TTC GAA ATG CTT TGA TGG CAG AGC 1980
  T   S   D   S   I   A   N   L   L   T   R   I   R   N   A   L   M   A   E   H
                                                                Eco RI
ATT TGT ACA TTG ATA TCG AGC ATA GTA AAA TGC TTG AAG CAA TAG TAA GAA TTC TCA AGC 2040
  L   Y   I   D   I   E   H   S   K   M   L   E   A   I   V   R   I   L   K   Q

AGC ACG GGT TCA TTG CTC ACT TTT TAG TAA AAG AAG AAA ATC GCA AAA GAC TAA TGA GAG 2100
  H   G   F   I   A   H   F   L   V   K   E   E   N   R   K   R   L   M   R   V

TCT TTT TGC GGT ACG GGG AAG ATC GTA GAC CTG TGA TTC ATG CTC TTA AGC GTG TGT CTA 2160
  F   L   R   Y   G   E   D   R   R   P   V   I   H   A   L   K   R   V   S   K
      Xba I
AAC CTT CTA GAA GGG TTT ATG TTT CTG CAG CAA AAA TTC CTT ATG TAT TTG GAA ATA TGG 2220
  P   S   R   R   V   Y   V   S   A   A   K   I   P   Y   V   F   G   N   M   G

GTA TTG CCG TTC TTT CGA CTC CTC AAG GGG TTT TAG AAG GCT CTG TAG CAA GGG CTA AGA 2280
  I   A   V   L   S   T   P   Q   G   V   L   E   G   S   V   A   R   A   K   N

ATG TTG GCG GCG AAT TGC TTT GTT TGG TTT GGT AGC AAA TTA AAA GAT TAG GAC GGT AAC 2340
  V   G   G   E   L   L   C   L   V   W   *  end Ctr S8e
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                    Xho I
GAA TGT CTC GTA AAG CTC GAG ACC CTA TTG TGC TTC CTC AAG GCG TAG AGG TCT CTA TTC 2400
 M   S   R   K   A   R   D   P   I   V   L   P   Q   G   V   E   V   S   I   Q
 start Ctr L6e

AAA ATG ATG AAA TCT CAG TAA AAG GTC CTA AAG GGT CTT TGA CGC AGG TAT TGG CTA AAG 2460
 N   D   E   I   S   V   K   G   P   K   G   S   L   T   Q   V   L   A   K   E

AAG TTG AGA TTG CCG TTA AAG GTA ATG AGG TGT TTG TTG CTC CTG CGG CTC ACG TTG TAG 2520
 V   E   I   A   V   K   G   N   E   V   F   V   A   P   A   A   H   V   V   D

ACA GAC CTG GTC GTA TGC AAG GGC TTT ATT GGG CCT TAA TAG CAA ATA TGG TCA AAG GTG 2580
 R   P   G   R   M   Q   G   L   Y   W   A   L   I   A   N   M   V   K   G   V

TCC ATA CTG GAT TTG AGA AGC GTT TAG AAA TGA TCG GAG TCG GCT TCA GAG CTG CAG TAC 2640
 H   T   G   F   E   K   R   L   E   M   I   G   V   G   F   R   A   A   V   Q

AAG GGT CCT TGT TAG ATC TGT CAA TAG GGG TTT CTC ACC CTA CAA AAA TGC CTA TTC CTA 2700
 G   S   L   L   D   L   S   I   G   V   S   H   P   T   K   M   P   I   P   T

CGG GAT TAG AAG TCT CTG TTG AGA AAA ACA CAT TGA TCT CCA TTA AAG GTA TCA ATA AGC 2760
 G   L   E   V   S   V   E   K   N   T   L   I   S   I   K   G   I   N   K   Q

AGT TAG TTG GAG AAT TTG CGG CTT GTG TTC GTG CAA AAC GCC CTC CAG AAC CAT ACA AAG 2820
 L   V   G   E   F   A   A   C   V   R   A   K   R   P   P   E   P   Y   K   G

GTA AAG GAA TTC GTT ACG AAA ACG AAT ATG TTC GTC GTA AGG CTG GGA AAG CAG CGA AAA 2880
 K   G   I   R   Y   E   N   E   Y   V   R   R   K   A   G   K   A   A   K   T

CTG GTA AAA AAT AGA GGG TAA AGT AGA GTC GAA CTA TGG AAA GCT CTT TAT ATA AGA AAA 2940
 G   K   K   *  end Ctr L6e                  M   E   S   S   L   Y   K   K   T
                                             start Ctr L18e

CTT CGG GGA AAG CTC GTA GAG CTT TAA GAG TGC GGA AAG CCT TAA AGG GAT GTT CTT TAA 3000
 S   G   K   A   R   R   A   L   R   V   R   K   A   L   K   G   C   S   L   K

AGC CCA GAT TAT CCG TTG TAA AGA CAA ATA AGC ATG TTT ATG TGC AGC TGA TTG ATG ATG 3060
 P   R   L   S   V   V   K   T   N   K   H   V   Y   V   Q   L   I   D   D   V

TTG AAG GGA AAA CTT TAG CAT TTA TTT CAA CTT TGG CTA AGG TTG CAA AAA CTT CTG GAT 3120
 E   G   K   T   L   A   F   I   S   T   L   A   K   V   A   K   T   S   G   L
                                    Hind III
TAA CTA GAA AAA ATC AGG ATA ATG CCA AAG CTT TGG GAA TAA AAA TTG CTG AAT TAG GGA 3180
 T   R   K   N   Q   D   N   A   K   A   L   G   I   K   I   A   E   L   G   K
                                                  Sac I
AAG GCC TTC AAG TAG ATC GAG TTG TTT TCG ATC GAG GAG CTC ATA AGT ATC ATG GTG TAG 3240
 G   L   Q   V   D   R   V   V   F   D   R   G   A   H   K   Y   H   G   V   V

TAG CTA TGG TTG CTG ATG GAG CCA GAG AGG GTG GAT TAC AGT TTT AAT GAA GGT TTA GAT 3300
 A   M   V   A   D   G   A   R   E   G   G   L   Q   F   *   *  end Ctr L18e

AAT GAC GCT ATC AAG AAA TTC TCA TAA GGA AGA TCA GCT GGA AGA GAA GGT TCT CGT CGT 3360
 M   T   L   S   R   N   S   H   K   E   D   Q   L   E   E   K   V   L   V   V
 start Ctr S5e

CAA CCG TTG TTG TAA GGT TGT TAA AGG AGG CCG TAA GTT TAG TTT TTC TGC GCT TAT TTT 3420
 N   R   C   C   K   V   V   K   G   G   R   K   F   S   F   S   A   L   I   L

AGT TGG CGA TAG AAA AGG GCG TTT AGG CTT CGG ATT TGC GAA AGC TAA CGA GCT AAC TGA 3480
 V   G   D   R   K   G   R   L   G   F   G   F   A   K   A   N   E   L   T   D

TGC CAT CCG TAA AGG TGG GGA TGC TGC TCG AAA AAA TCT TGT CTC TAT CAA TTC TCT TGA 3540
 A   I   R   K   G   G   D   A   A   R   K   N   L   V   S   I   N   S   L   E
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GGG AGG ATC TAT TCC TCA TGA GGT TCT TGT CAA TCA TGA TGG AGC AGA GCT TCT GTT AAA 3600
 G   G   S   I   P   H   E   V   L   V   N   H   D   G   A   E   L   L   L   K

ACC TGC TAA GCC AGG AAC CGG AAT CGT TGC AGG ATC TCG TAT TCG GTT GAT TTT AGA GAT 3660
 P   A   K   P   G   T   G   I   V   A   G   S   R   I   R   L   I   L   E   M
                                                Bam HI
GGC CGG GGT AAA GGA CAT TGT AGC AAA GAG TTT AGG ATC CAA TAA TCC TAT GAA TCA GGT 3720
 A   G   V   K   D   I   V   A   K   S   L   G   S   N   N   P   M   N   Q   V

TAA AGC GGC TTT TAA AGC TCT CCT GAC ACT CTC TTG TAA AGA TGA TAT TAT GAA AAG GAG 3780
 K   A   A   F   K   A   L   L   T   L   S   C   K   D   D   I   M   K   R   R

AGC CGT TAT CAA TGA TTA AGT TAG AGT GTT TAC AAG ATC CTT CGC CTC GTA AGC GAA GAA 3840
 A   V   I   N   D   *  end Ctr S5e
              M   I   K   L   E   C   L   Q   D   P   S   P   R   K   R   R   T
              start Ctr L15e

CGA AAC TCT TGG GCC GAG GAC CTT CTT CTG GTC ACG GGA AAA CAA GTG GTC GAG GAC ACA 3900
 K   L   L   G   R   G   P   S   S   G   H   G   K   T   S   G   R   G   H   K

AAG GGG ACG GTA GCC GTT CTG GAT ACA AGA GAC GTT TCG GAT ATG AAG GGG GAG GCG TAC 3960
 G   D   G   S   R   S   G   Y   K   R   R   F   G   Y   E   G   G   G   V   P

CTT TAT ACA GAA GAG TTC CTA CAC GAG GAT TTT CTC ATA AAC GCT TTG ATA AAT GTG TTG 4020
 L   Y   R   R   V   P   T   R   G   F   S   H   K   R   F   D   K   C   V   E

AAG AAA TCA CAA CAC AAC GTT TGA ATG AGA TTT TTG ACA ATG GCG CAG AAG TAT CTT TGG 4080
 E   I   T   T   Q   R   L   N   E   I   F   D   N   G   A   E   V   S   L   E
Hind III
AAG CTT TAA AAG AAA GAA AAG TTA TCC ATA GAG AGA CTT CTC GTG TTA AAG TAA TCC TTA 4140
 A   L   K   E   R   K   V   I   H   R   E   T   S   R   V   K   V   I   L   K

AAG GAG CTC TGG ATA AGA AAT TAG TCT GGA AAG ATG CTG CAA TAG TGC TGT CAG AAG GAG 4200
 G   A   L   D   K   K   L   V   W   K   D   A   A   I   V   L   S   E   G   V
                                                     SD
TAA AAA GTC TTA TCG AGG CTG TTT AAC TAG AAC TTT TAG GTA AAG TTT ATG GCT ACA TTG 4260
 K   S   L   I   E   A   V   *  end Ctr L15e                    M   A   T   L
                                                                start Ctr SecYe

CGA CAA GTG TTT TCG ATT TCC GAA CTG CGA CAA AAA ATA TTT TTC ACA TTT TCC TTG CTT 4320
R   Q   V   F   S   I   S   E   L   R   Q   K   I   F   F   T   F   S   L   L

GCA TTA TGT AGA ATC GGG GTG TTT ATC CCT GTG CCT GGA ATT AAC GGA GAC CGC GCC GTA 4380
A   L   C   R   I   G   V   F   I   P   V   P   G   I   N   G   D   R   A   V

GCC TAC TTT AAC CAA TTG CTG GGG TCT AGC CGG GGT TTG TTT CAG TTA GCT GAC ATT TTT 4440
A   Y   F   N   Q   L   L   G   S   S   R   G   L   F   Q   L   A   D   I   F

TCT GGG GGA GCT TTT GCT CAA ATG ACG GTA ATA GCT CTT GGA GTT GTT CCG TAC ATC TCG 4500
S   G   G   A   F   A   Q   M   T   V   I   A   L   G   V   V   P   Y   I   S

GCT TCA ATC ATT GTA CAG CTT CTT GTC GTC TTT ATG CCG ACT CTG CAA AGA GAA ATG CGA 4560
A   S   I   I   V   Q   L   L   V   V   F   M   P   T   L   Q   R   E   M   R

GAG TCG CCG GAT CAA GGG AAG CGT AAA TTA GGA CGA ATG ACA CGG CTT TTT ACT CTT GTT 4620
E   S   P   D   Q   G   K   R   K   L   G   R   M   T   R   L   F   T   L   V

CTA GCC TGT GTA CAG TCT TTG CTT TTT GCA AAA TTT GCT CTG CGA ATG AAT CTT GTT GTT 4680
L   A   C   V   Q   S   L   L   F   A   K   F   A   L   R   M   N   L   V   V

CCA GGG ATT GTT TTG CCA GCA ATG TTG TCC TTA AAG CTG TTT GGG GTG CCT TGG GTA TTT 4740
P   G   I   V   L   P   A   M   L   S   L   K   L   F   G   V   P   W   V   F
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TAT TTG ACA ACT GTT GTG GTT ATG ACA ACA GGG ACT CTT TTA CTT ATG TGG GTT GGA GAG 4800
Y   L   T   T   V   V   V   M   T   T   G   T   L   L   L   M   W   V   G   E

CAA ATA TCT GAC AAA GGG ATT GGT AAT GGA ATC AGT TTG ATC ATT ACT CTC GGG ATA TTA 4860
Q   I   S   D   K   G   I   G   N   G   I   S   L   I   I   T   L   G   I   L
                                                                         Bam HI
GCC TCT TTT CCT TCC GTT TTA GGG TCT ATA TTT AAC AAG TTA AAT TTG GGA TCT CAG GAT 4920
A   S   F   P   S   V   L   G   S   I   F   N   K   L   N   L   G   S   Q   D

CCT TCT GAA TTT GGA ATC GTT TCG CTT TTA ATT CTT TGC GCG GTT TTT GTC TTT GTT CTT 4980
P   S   E   F   G   I   V   S   L   L   I   L   C   A   V   F   V   F   V   L

ATA GCA ACT GTG CTC ATT ATT GAA GGT GTA AGA AAG ATT CCT GTT CAG CAT GCA CGT AGA 5040
I   A   T   V   L   I   I   E   G   V   R   K   I   P   V   Q   H   A   R   R

ATT ATT GGA AGG AGA GAG GTT GTA GGA GGG GGA TCG TAT CTT CCT TTG AAA GTG AAT TAT 5100
I   I   G   R   R   E   V   V   G   G   G   S   Y   L   P   L   K   V   N   Y

GCT GGA GTA ATC CCG GTT ATT TTT GCT TCC TCT TTA CTC ATG TTC CCA GCT ACG ATT GGG 5160
A   G   V   I   P   V   I   F   A   S   S   L   L   M   F   P   A   T   I   G

CAG TTT CTT TCC TCG GAA TCT TCT TGG TTG AAA CGC ATT GCG ACT ATG CTG TCC CCA GGT 5220
Q   F   L   S   S   E   S   S   W   L   K   R   I   A   T   M   L   S   P   G

AGT GTG GCG TAT TCC ATT TTT TAT GTG TTG CTT ATT ATA TTT TTC ACT TAT TTT TGG ACA 5280
S   V   A   Y   S   I   F   Y   V   L   L   I   I   F   F   T   Y   F   W   T

GCT ACG CAG TTC CGT CCA GAG CAA ATA GCT TCT GAA ATG AAG AAA AAT GGA GCG TTT ATT 5340
A   T   Q   F   R   P   E   Q   I   A   S   E   M   K   K   N   G   A   F   I

CCT GGC ATT AGA CAA GGG AAA CCA ACC CAG ACC TAT CTT GAA TAC ACA ATG AAT AGG GTA 5400
P   G   I   R   Q   G   K   P   T   Q   T   Y   L   E   Y   T   M   N   R   V

ACT TTG CTA GGA GCT GTA TTT TTA GCT GTT GTA GCT ATA TTA CCT TCC GTT TTG GGA AGG 5460
T   L   L   G   A   V   F   L   A   V   V   A   I   L   P   S   V   L   G   R

ATT TTG AGA GTT GAC GCG AAC GTT AGC TAC TTT TTG GGT GGA ACA GCC ATG CTG ATC GTA 5520
I   L   R   V   D   A   N   V   S   Y   F   L   G   G   T   A   M   L   I   V

GTC GGA GTC ATC TTG GAC ACG ATG AAG CAA ATT GAT GCT TTC CTT TTA GTC CGG CGT TAT 5580
V   G   V   I   L   D   T   M   K   Q   I   D   A   F   L   L   V   R   R   Y
                                                      -35
GAC GGA GTT TTA AAG AAA GAC CGC CCC AAA GGA AGA CCT TGA AAA ATA ACA ATT TTT GAC 5640
D   G   V   L   K   K   D   R   P   K   G   R   P   *  end Ctr SecYe
    -10                 +1       SD
CTA AGA TGC TTA TAC TAC TTT AAG GGA GGC CCT TCG TAT GCC GCG CAT CAT TGG AAT AGA 5700
                                                M   P   R   I   I   G   I   D
                                                start Ctr S13e

TAT TCC TGC GAA AAA GAA ATT AAA AAT AAG TCT TAC ATA TAT TTA TGG AAT AGG GCC AGC 5760
 I   P   A   K   K   K   L   K   I   S   L   T   Y   I   Y   G   I   G   P   A
                                                                 Pst I
TCT TTC TAA AGA GAT TAT TGC TAG ATT GCA GTT GAA TCC CGA AGC TAG AGC TGC AGA GTT 5820
 L   S   K   E   I   I   A   R   L   Q   L   N   P   E   A   R   A   A   E   L

GAC TGA GGA AGA GGT TGG TCG ACT AAA CGC TCT TTT ACA GTC GGA TTA CGT TGT TGA AGG 5880
 T   E   E   E   V   G   R   L   N   A   L   L   Q   S   D   Y   V   V   E   G

GGA TTT GCG CCG TCG TGT GCA ATC TGA TAT CAA ACG TCT GAT TAC TAT CCA TGC TTA TCG 5940
 D   L   R   R   R   V   Q   S   D   I   K   R   L   I   T   I   H   A   Y   R

TGG ACA AAG ACA TAG ACT TTC TTT GCC TGT TCG TGG TCA GAG AAC AAA AAC AAA TTC TCG 6000
 G   Q   R   H   R   L   S   L   P   V   R   G   Q   R   T   K   T   N   S   R
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                                                                          SD
CAC GCG TAA GGG TAA ACG TAA AAC TAT TGC AGG TAA GAA GAA ATA ATA ATT TTT AGG AGA 6060
 T   R   K   G   K   R   K   T   I   A   G   K   K   K   *   *   end Ctr S13e

GAG TGT TTT GGT TAA AAA TCA AGC GCA AAA AAG AGG CGT AAA AAG AAA ACA AGT AAA AAA 6120
         L   V   K   N   Q   A   Q   K   R   G   V   K   R   K   Q   V   K   N
        start Ctr S11e

CAT TCC TTC GGG CGT TGT CCA TGT TAA GGC TAC TTT TAA TAA TAC AAT TGT AAC CAT AAC 6180
 I   P   S   G   V   V   H   V   K   A   T   F   N   N   T   I   V   T   I   T

AGA CCC TGC TGG TAA TGT GAT TTC ATG GGC TTC TGC TGG GAA AGT TGG TTA TCC TGG TTC 6240
 D   P   A   G   N   V   I   S   W   A   S   A   G   K   V   G   Y   P   G   S

TCG TAA ATC TTC AGC ATT TGC TGC GAC GGT TGC CGC TCA AGA TGC TGC TAA GGC TGC TAT 6300
 R   K   S   S   A   F   A   A   T   V   A   A   Q   D   A   A   K   A   A   M

GAG TTC TGG ATT AAA AGA AGT TGA AGT AGG CTT AAA AGG AAC TGG TGC AGG GCG GGA ATC 6360
 S   S   G   L   K   E   V   E   V   G   L   K   G   T   G   A   G   R   E   S

TGC TGT GCG AGC GCT AAT TTC TTC TGG GCT TAT CGT TTC CGT TAT CCG AGA TGA AAC TCC 6420
 A   V   R   A   L   I   S   S   G   L   I   V   S   V   I   R   D   E   T   P
                         Sal I                                             SD
CGT CCC TCA TAA CGG GTG TCG ACC AAG AAA ACG ACG AAG AGT GTA GTT ATA GCT AAG GAG 6480
 V   P   H   N   G   C   R   P   R   K   R   R   R   V   *   end Ctr S11e

TTT GGG ATG TCG GAT AGT TCA CAC AAT TTA CTT TAT AAC AAA TTT GAG TTG CCT GAA TCG 6540
         M   S   D   S   S   H   N   L   L   Y   N   K   F   E   L   P   E   S
        start Ctr ae
                    Eco NI
GTG AAG ATG TCT CCT GTG GAA GGG GCT GTT GGC AGC ATT GAT AAA GTA GCT CGA TTT GTT 6600
 V   K   M   S   P   V   E   G   A   V   G   S   I   D   K   V   A   R   F   V

GCA GAT CCC TTG GAA AAA GGG ATG GGG CAC ACC TTG GGA AGC GCC TTG CGA CGT GCT CTG 6660
 A   D   P   L   E   K   G   M   G   H   T   L   G   S   A   L   R   R   A   L

TTA ATC GGC TTG GAA GCT CCT GCT ATT GTC TCT TTC TCT ATG ACA GGA GTT TTG CAC GAA 6720
 L   I   G   L   E   A   P   A   I   V   S   F   S   M   T   G   V   L   H   E

TAT ATG GCG GTA GAG GGG ATC ATT GAA GAT GTT ACC AAT ATG CTT TTG AAT TTG AAA GGT 6780
 Y   M   A   V   E   G   I   I   E   D   V   T   N   M   L   L   N   L   K   G

TCG TTG CTT AAA AAG TAT CCT CTA CAA GAT TGT GAA GGT GGA AGA TGC TCT CAA AAG TTA 6840
 S   L   L   K   K   Y   P   L   Q   D   C   E   G   G   R   C   S   Q   K   L
                             Nsi I
CGA GCT ACG ATT TCT GTT GAT GCA TCC GAT TTA GCT GCT GCT GGT GGG CAG AAG GAA GTT 6900
 R   A   T   I   S   V   D   A   S   D   L   A   A   A   G   G   Q   K   E   V

ACT TTA GGA GAT TTG CTA CAA GAA GGA ACT TTT GAA GCG GTC AAT CCT GAG CAC GTA ATT 6960
 T   L   G   D   L   L   Q   E   G   T   F   E   A   V   N   P   E   H   V   I

TTT ACG GTC ACG CGT CCA ATG CAA CTT GAG GTT ATG TTG CGA GTT GCT TTT GGT AGA GGA 7020
 F   T   V   T   R   P   M   Q   L   E   V   M   L   R   V   A   F   G   R   G

TAC TCT CCT TCT GAA AGA ATC GTT CTT GAA GAA AGA GGC ATG AAT GAG ATC GTT TTA GAT 7080
 Y   S   P   S   E   R   I   V   L   E   E   R   G   M   N   E   I   V   L   D

GCG GCA TTC TCT CCT GTT GTT CTG GTT AAC TAT TTT GTT GAA GAC ACC CGC GTT GGA CAA 7140
 A   A   F   S   P   V   V   L   V   N   Y   F   V   E   D   T   R   V   G   Q

GAT ACA GAT TTC GAT CGT TTA GTG TTG CAA GTG GAA ACC GAT GGT CGT GTG GCT CCT AAA 7200
 D   T   D   F   D   R   L   V   L   Q   V   E   T   D   G   R   V   A   P   K
```

```
GAA GCT GTA GCT TTT GCT ACA CAG ATT TTG AGT AAG CAT TTT TCT GTT TTC GAA AAA ATG 7260
E   A   V   A   F   A   T   Q   I   L   S   K   H   F   S   V   F   E   K   M

GAC GAG AAG AGA ATC GTT TTT GAA GAA GCA ATC TCT GTA GAG AAA GAA AAC AAA GAC GAT 7320
D   E   K   R   I   V   F   E   E   A   I   S   V   E   K   E   N   K   D   D
                                                            Rsa I
ATT CTT CAT AAA TTG GTT TTA GGC ATT AAT GAG ATA GAA CTT TCT GTA CGC TTG ATA CGA 7380
I   L   H   K   L   V   L   G   I   N   E   I   E   L   S   V   R   L   I   R

TCT ACA AAT TGT TTA TCT AAT GCC AAT ATC GAA ACG ATA GGG GAA TTG GTA ATT ATG CCA 7440
S   T   N   C   L   S   N   A   N   I   E   T   I   G   E   L   V   I   M   P

GAG CCT CGT CTG TTA CAA TTT AGA AAT TTC GGG AAG AAG TCT CTC TGC GAG ATT AAG AAT 7500
E   P   R   L   L   Q   F   R   N   F   G   K   K   S   L   C   E   I   K   N

AAA CTG AAA GAA ATG AAA TTA GAG TTA GGC ATG GAC CTC AGC CAG TTT GGT GTT GGT CTG 7560
K   L   K   E   M   K   L   E   L   G   M   D   L   S   Q   F   G   V   G   L

GAT AAC GTT AAA GAA AAA ATG AAG TGG TAT GCC GAA AAA ATT CGG TCG AGT AAA AAT ACC 7620
D   N   V   K   E   K   M   K   W   Y   A   E   K   I   R   S   S   K   N   T
                                                                  Rsa I
AAG GCA TAA AAG AGA TAT GCA ACA CGC TAG AAA AAA ATT TAG GGT TGG TCG TAC TCG TCT 7680
K   A   *   K   R   Y   A   T   R   *  end Ctr ae

CAT AAC CGT TGC ATG TTG GCT AAT ATG TTG AAG TCT TTG ATT CAC AAT GAA AGA ATA GAG 7740
                M   L   A   N   M   L   K   S   L   I   H   N   E   R   I   E
                start Ctr L17e

ACT ACA TTG CCT AAA GCC AAA GAG TTG CGT CGG CAT GCA GAC AAA ATG ATT ACT TTA GCT 7800
T   T   L   P   K   A   K   E   L   R   R   H   A   D   K   M   I   T   L   A

AAG AAA AAT ACC TTA GCT GCA AGA AGA TTA GCT GTA GGG CGT CTT ATG GTC AGA TAT AAT 7860
K   K   N   T   L   A   A   R   R   L   A   V   G   R   L   M   V   R   Y   N

ACG TTG ACT AGC AAA GAG GCT CGC CAA GTT AAA GCT GGA GAT TTG TCT GCT TAT AAT GTT 7920
T   L   T   S   K   E   A   R   Q   V   K   A   G   D   L   S   A   Y   N   V

GAT AGA AGA GTC ATT GGG AAG TTA TTT GAT GTG TTA GCA ACC AGG TTT TCT TCG AGA AAT 7980
D   R   R   V   I   G   K   L   F   D   V   L   A   T   R   F   S   S   R   N

CGC GGG TAT ACG CGC ATT TTG AAG TTG CAA AAT AGG GTT GGT GAT AAT GCT CAA AAG TGT 8040
R   G   Y   T   R   I   L   K   L   Q   N   R   V   G   D   N   A   Q   K   C

                -------->      <--------
ATC ATA GAA TTT TTA GCA TAG TGA TGC TAA TTT TTC GAA AAC ACT GAC TAC CTG GGA TTT 8100
I   I   E   F   L   A   *   *  end Ctr L17e

AGC AAT GAG AAT TGT GAT TAA TGG TTT TGG ACG GAT TGG GCG ATT AGT TTT AAG ACA GAT 8160
     M   R   I   V   I   N   G   F   G   R   I   G   R   L   V   L   R   Q   I
     start Ctr G3Pe
             Eco RI
TCT GAA AAG GAA TTC TCC CAT AGA AGT TGT AGC TAT TAA TGA TTT AGT CGC AGG AGA TCT 8220
 L   K   R   N   S   P   I   E   V   V   A   I   N   D   L   V   A   G   D   L

TTT AAC ATA TTT ATT TAA ATA TGA TTC CAC ACA CGG ATC TTT CGC TCC TCA AGC AAC ATT 8280
 L   T   Y   L   F   K   Y   D   S   T   H   G   S   F   A   P   Q   A   T   F

TTC GGA TGG ATG TTT GGT TAT GGG AGA AAG AAA GAT CCG TTT CTT AGC GGA AAA AGA CGT 8340
 S   D   G   C   L   V   M   G   E   R   K   I   R   F   L   A   E   K   D   V

     Hind III
TCA AAA GCT T                                                                   8350
 Q   K   L
```

has a TGA stop codon. Also, the intergenic regions in this gene cluster differed from one another in both length and sequence.

The organization of r-protein genes in *C. trachomatis* appears similar to that of the *S10-spc-α* operons of *E. coli* except for the absence of the genes for the r-proteins CtrS14e, CtrL30e, CtrXe and CtrS4e in *C. trachomatis*. The gene order in the cluster was found to be CtrL16e, CtrL29e, CtrS17e, CtrL14e, CtrL24e, CtrL5e, CtrL8e, CtrL6e, CtrL18e, CtrS5e, CtrL15e, CtrSecYe, CtrS13e, CtrS11e, Ctrαe and CtrL17e. The first three genes at the 5' end in this cluster are very similar to the last three genes of the *S10* operon. A 16-bp intergenic region between the end of the CtrS17e coding sequence (*S10* operon) and the beginning of the CtrL14e coding sequence (*spc* operon) was found; it did not include any apparent transcription termination sequences. In two regions, CtrL29e-CtrS17e and CtrS5e-CtrL15e, the ORFs overlap for a few bases. No rho-independent terminator sequence was found within the 55 bp intergenic region between *secY* and *S13*. A possible ribosome binding site was located just 7 bp upstream of the ATG translation start site. Except for a 55-bp inter-cistronic region between *secY* and *S13* genes and a 63-bp region between *α* and *L17*, the α operon genes are closely linked. An internal promoter sequence upstream of *S13* showing identity among 5 out of 6 positions when compared to the *E. coli* consensus sequence was identified. The sequence distal to α ORF revealed a 7-bp dyad with the potential of forming a stem and loop structure followed by 5 uridine residues.

The organization of the α operon gene cluster in *C. trachomatis* is similar but not identical to that either in *E. coli* or *B. subtilis*. The gene order in the cluster was found to be S13, S11, α and L17. The sequence downstream of the L17 gene revealed another ORF whose amino acid sequence was similar to that of *E. coli* glyceraldehyde-3-phosphate dehydrogenase. A 55-bp intergenic region was found between *secY*, the last gene of *spc* operon, and *S13*, the first gene of α operon. This region does not contain any obvious rho-dependent or rho-independent terminator sequences when analyzed by computer. No overlapping translational stop and start codons were found in the α operon region.

III.1.3 Analysis of the deduced amino acid sequence of r-proteins. Alignments of the amino acid sequences of the 11 r-proteins of the *S10 -spc* gene cluster from *C. trachomatis* and *E. coli*, respectively, are shown in Fig. 3. The percent sequence identities vary from 57% for CtrL14e to 28% for CtrL29e and CtrL24e. Consideration of conservative substitution raises the homology to 77% for CtrL18e and approximately 50% for CtrS8e. In the case of CtrL18e, the homology was more marked toward its carboxyl-terminal region. Table 1 shows a comparison of the total numbers of amino acid residues of each protein from *C. trachomatis* and *E. coli*. With the exception of CtrL5e, which is 24 amino acid residues short, no significant length differences were observed among other r-proteins.

FIG. 3. Comparison of the deduced amino acid sequence of *C. trachomatis* (Ctr) *S10-spc* operons with the *E. coli* (Eco) r-protein gene cluster. The single-letter amino acid code is used. Dashes indicate gaps introduced to optimize alignment, while vertical lines indicate identical amino acids. Numbers to the right refer to the percent amino acid identity between Ctr and Eco r-proteins.

```
                                                                        Identity(%)

Eco L16  MLQPKRTKFRKMHKGRNRGLAQGTDVSFGSFGLKAVGRGRLTARQIEAARRAMTRAVKRQ      38

Ctr L16e                            APDHWVAVVRPGRILFEVANVSKEDAQDALRRA
                                        |||   ||  | |   |  | |  |   |
Eco L16  GKIWIRVFPDKPITEKPLAVRMGKGKGNVEYWVALIQPGKVLYEMDGVPEELAREAFKLA

Ctr L16e AAKLGIRTRFVKRVERV
         |||| | | ||
Eco L16  AAKLPIKTTFVTKTVM


Ctr L29e MGAKKNLLAELREKSSEELDEFIRDNKKALFALRAEAALQNKVVKTHQFSLYKKSIARAL      28
                  |||||| |||           | ||  ||        |         ||
Eco L29       MKAKELREKSVEELNTELLNLLREQFNLRMQAA-SGQLQQSHLLKQVRRDVARVK

Ctr L29e IIKQEKKGRVHG
             || |
Eco L29  TLLNEKAGA


Ctr S17e MASDVRGRRKTKIGVVVSSKMEKTVVVRVERVYSHPQYAKVVRDSSKYYAHNELDVKE-G      41
                   |  | ||| ||||  ||  ||   || | |      |   | |      |
Eco S17       TDKIRTLQGRVVSDKMEKSIVVAIERFVKHPIYGKFIKRTTKVHVHDENNECGIG

Ctr S17e DTV-RIQETRPLSKTKRWRVVGRVN
         | |  | | ||||||| |  |  |
Eco S17  DVVCEIRECRPLSKTKSWTLVRVVEKAVL


Ctr L14e MIQQESQLKVADNTGAKKVKCFKVLGGSRRRYATVGDVIVCSVRDIEPDSSVKKGDVVKA      57
         |||    | |||| ||  | | |||||| |||| ||| |        |   |||||| ||
Eco L14  MIQEQTMLNVADNSGARRVMCIKVLGGSHRRYAGVGDIIKITIKEAIPRGKVKKGDVLKA

Ctr L14e VIVRTRNDIHRKDGSTLRFDTNSCVIIDDKG-NPKGTRIFGPVAREIRDRGFVKISSLAP
         | |||     | |||  ||| | ||        | |||||||| || |   | || ||||
Eco L14  VVVRTKKGVRRPDGSVIRFDGNACVLLNNNSEQPIGTRIFGPVTRELRSEKFMKIISLAP

Ctr L14e EVI
         ||
Eco L14  EVL


Ctr L24e MKRRSVCVGDTVYVLAGNDKGKQGKVLRCL-KDKVVVEGINVRVKNIKRSQE-NPKGKRI      28
                  | | || | |||| |||   |   || |||||   |  |     |  |
Eco L24   MAAKIRRDDEVIVLTGKDKGKRGKVKNVLSSGKVIVEGINLVKKHQKPVPALNQPGGIV

Ctr L24e NIEAPLHISNVRLSIDNQPARLFVKVTEKGRELWNKHSDGSSSLYRLVRERKG
           ||    |||            |                |
Eco L24  EKEAAIQVSNVAIFNAATGKADRVGFRFEDGKKVRFFKSNSETIK


Ctr L5e                           MQIPVLKKIVISMGLAEAAKDKNLFQAHLEELAVI      52
                                  || |   ||   ||  ||  || |       || |
Eco L5   MAKLHDYYKDEVVKKLMTEFNYNSVMQVPRVEKITLNMGVGEAIADKKLLDNAAADLAAI

Ctr L5e  SGQKPLVTRAKNSIAGFKLREGQGIGAKVTLRGIRMYDFMDRFCNIVSPRIRDFRGFSCK
         |||||| | |  | |||| | |  || |||||| ||  |  |   |  |||||||| | |
Eco L5   SGQKPLITKARKSVAGFKIRQGYPIGCKVTLRGERMWEFFERLITIAVPRIRDFRGLSAK

Ctr L5e  G-DGRGCYSLGLDDQQIFPEVDLDAVKRSQGMNITWVTTAQTDAECLTLLECMGLRFKKAQ
           |||| || |   | |||| | | | |  |  ||  |||  | |   ||      | |
Eco L5   SFDGRGNYSMGVREQIIFPEIDYDKVDRVRGLDITITTTAKSDEEGRALLAAFDFPFRK
```

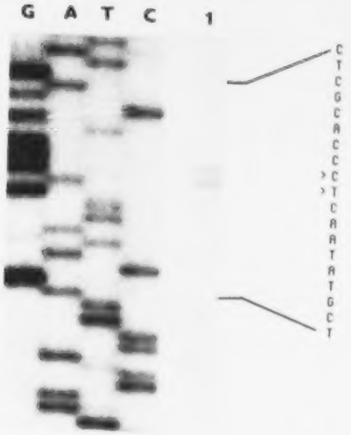
```
Ctr S8e   MGMTSDSIANLLTRIRNALMAEHLYIDIEHSKMLEAIVRILKQHGFIAHFLVKEENRKRL      37
               | ||  ||||||   |         ||   ||   ||  |||  | | |   |
Eco S8     MSMQDPIADMLTRIRNGQAANKAAVTMPSSKLKVAIANVLKEEGFIEDFKV-EGDTKPE

Ctr S8e   MRVFLRYGEDRRPVIHALKRVSKPSRRVYVSAAKIPYVFGNMGIAVLSTPQGVLEGSVAR
              | |      |     ||| |  | |      | |    |||| ||  ||     ||
Eco S8    LELTLKYFQG-KAVVESIQRVSRPGLRIYKRKDQLPKVMAGLGIAVVSTSKGVMTDRAAR

Ctr S8e   AKNVGGELLCLVW
              |||  | |
Eco S8    QAGLGGEIICYVA


Ctr L6e   MSRKARDPIVLPQGVEVSIQNDEISVKGPKGSLTQVLAKEVEIAVKGNEVFVAPAAHVVD      42
          ||| |  | | | || | |    |  ||  | ||  |   ||     |     |     |
Eco L6    MSRVAKAPVVVPAGVDVKINGQVITIKGKNGELTRTLNDAVEVKHADNTLTFGPRDGYAD

Ctr L6e   RPGRMQGLYWALIANMVKGVHTGFEKRLEMIGVGFRAAVQGSLLDLSIGVSHPTKMPIPT
                |   ||   || ||   | | |   ||| |||| |    || | |||     |
Eco L6    GWA-QAGTARALLNSMVIGVTEDFTKKLQLVGVGYRAAVKGNVINLSLGFSHPVDHQLPA

Ctr L6e   GLEVSVEKNTLISIKGINKQLVGEFAACVRAKRPPEPYKGKGIRYENEYVRRKAGKAAKTGKK
          |        | |  ||  ||  |  ||  || | |||||||| ||  | || |  |
Eco L6    GITAECPTQTEIVLKGADKQVIGQVAADLRAYRRPEPYKGKGVRYADEVVRTKEAKKK


Ctr L18e  MESSLYKKTSGKARRALRVRKALKGCSLKPRLSVVKTNKHVYVQLIDDVEGKTLAFISTL      36
                 | |   ||| | |  |       || |  |  | | | |       |   ||
Eco L18        MDKKSARIRRATRARRKLQELG-ATRLVVHRTPRHIYAQVIAPNGSEVLVAASTV

Ctr L18e  AKVAKTSGLTRKNQDNAKALGIKIAELGKGLQVDRVVFDRGAHKYHGVVAMVADGAREGG
           |          | | | | |   ||         | |||    ||| |   || ||| |
Eco L18   EKAIAEQLKYTGNKDAAAAVGKAVAERALEKGIKDVSFDRSGFQYHGRVQALADAAREAG

Ctr L18e  LQF
          |||
Eco L18   LQF


Ctr S5e   MTLSRNSHKEDQLEEKVLVVNRCCKVVKGGRKFSFSALILVGDRKGRLGFGFAKANELTD      40
                      | ||   |||  | ||||| ||| ||  |||  || |||  || |
Eco S5       AHIEKQAGELQEKLIAVNRVSKTVKGGRIFSFTALTVVGDGNGRVGFGYGKAREVPA

Ctr S5e   AIRKGGDAARKNLVSINSLEGGSIPHEVLVNHDGAELLLKPAKPGTGIVAGSRIRLILEM
          || |    || |      |  |   | |   | |      ||  |||| ||   |  ||
Eco S5    AIQKAMEKARRNMINV-ALNNGTLQHPVKGVHTGSRVFMQPASEGTGIIAGGAMRAVLEV

Ctr S5e   AGVKDIVAKSLGSNNPMNQVKAAFKALLTLSCKDDIMKRRAVIND
          |||    ||  || || | | |    |            |
Eco S5    AGVHNVLAKAYGSTNPINVVRATIDGLENMNSPEMVAAKRGKSVEEILGK


Ctr L15e  MIKLECLQDPSPRKRRTKLLGRGPSSGHGKTSGRGHKGDGSRSGYKRRFGYEGGGVPLYR      38
             |  |      |   | ||||  || ||| ||||||  ||||   | | |||  ||||
Eco L15    MRLNTLSPAEGSKKAGKRLGRGIGSGLGKTGGRGHKGQKSRSGGGVRRGFEGGQMPLYR

Ctr L15e  RVPTRGFSHKRFDKCVEEITTQRLNEIFDNGAEVSLEALKERKVIHRETSRVKVILKGAL
          | |  ||          ||    |      |  | |  ||    |       |||| |
Eco L15   RLPKFGFTSRK-AAITAEIRLSDLAKV--EGGVVDLNTLKAANIIGIQIEFAKVILAGEV

Ctr L15e  DKKLVWKDAAIVLSEGVKSLIEAV
                         |    |||
Eco L15   TTPVT--VRGLRVTKGARAAIEAAGGKIEE
```

TABLE 1. Comparison of *S10-spc* r-proteins of *C. trachomatis* and *E. coli*

Protein	No. of amino acid residues	
	E. coli	*C. trachomatis*
L29	63	72
S17	84	83
L14	123	122
L24	104	111
L5	179	155
S8	130	133
L6	177	183
L18	117	123
S5	167	165
L15	144	144

FIG. 4. Identification of the transcription start site of the *spc* operon by primer extension analysis. (A) The 5' end of the transcript is a doublet located 162 nucleotides upstream of the CtrL14e translation codon. A 19-mer synthetic oligonucleotide was annealed to total RNA isolated from the EB-infected HeLa cells at 36 hours postinfection (lane 1). The DNA sequencing ladders derived from the same primer are shown with GAT and C reactions from left to right. A complementary sequence is written on the right side of the sequencing ladder. Two arrows indicate the start sites. (B) Nucleotide sequence of the *spc* operon promoter region. The transcription start site is indicated as +1. Both -10 and -35 promoter sequences are labeled above the DNA sequence, and their nucleotides are underlined.

A


G
A
T
C
1
C
T
C
G
C
A
C
C
> C
> T
C
A
A
T
A
T
G
C
T

B

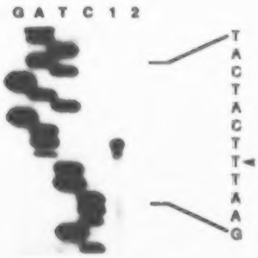
```
420                 -35                               -10
 CATCAAAAATGGAAAAAAACTGTTGTTGTTCGAGTCGAAAGGGTATACTCG
470     +1
 CACCCTCAATATGCTAAGGTGGTTAGGGATTCTAGCAAGTATTATGCGCA
```

III.1.4 Transcription and promoter studies. To study the temporal regulation of the *spc* and *S10* operon gene transcripts, total cellular RNA was isolated from *C. trachomatis*-infected HeLa cells at 21 and 36 h post infection and Northern blotted. Specific transcripts were detected by using a ^{32}P-labeled 939 bp *Hin*dIII fragment (nt 3174-4086) as a probe. This fragment contains the genes for r-protein CtrS5e and part of CtrL18e and CtrL15e. Only one transcript of approximately 5 kb was observed at both time points. To locate the transcription start site of the *spc* operon precisely, I analyzed the products of primer extension experiments using a 19-mer synthetic oligonucleotide (5' TCA CAT CAC CGA CCG TTG C 3') complementary to the coding strand from nucleotide 750 to 732 of the 8350-bp DNA sequence (Fig. 2). The transcript is initiated at a cytosine residue 162 nucleotides upstream of the CtrL14e start site, the first gene of the *spc* operon (Fig. 4). This cytosine residue is designated as +1 in the sequence. The cytosine was arbitrarily chosen instead of thymidine as the probable start site since there appears to be no difference in the intensity of bands corresponding to the two initiation sites. Inspection of the sequence upstream of the transcription start site identified -10 (TATACT) and -35 (CTGTTG) sequences within r-protein CtrS17e, which represents the end of the *S10* operon.

To locate the transcription start site of the α operon precisely, I analyzed the products of primer extension experiments using a 26-mer synthetic oligonucleotide (5' CGC AGG AAT ATC TAT TCC AAT GAT GC 3') complementary to the coding strand from nucleotide 5710

FIG. 5. Identification by primer extension analysis of the transcription start site of the *α* operon. (A) The 5' end of the transcript is located 18 nucleotides upstream of the CtrS13e initiation codon. A 26-mer synthetic oligonucleotide was annealed to total RNA from infected cells (lane 1) or uninfected 36-h-old HeLa cells (lane 2). The DNA sequencing ladders derived from the same primer are shown with G, A, T, and C reactions from left to right. A complementary sequence is written on the right side of the sequencing ladder. The arrow indicates the start site. (B) Nucleotide sequence of the α operon promoter region. The transcription start site is indicated as +1. Both -10 and -35 promoter sequences are labeled above the DNA sequence, and their nucleotides are underlined. The numbers indicate the corresponding nucleotide residues in Fig. 2.

A


G A T C 1 2
T
A
C
T
A
C
T
T ◄
T
A
A
G

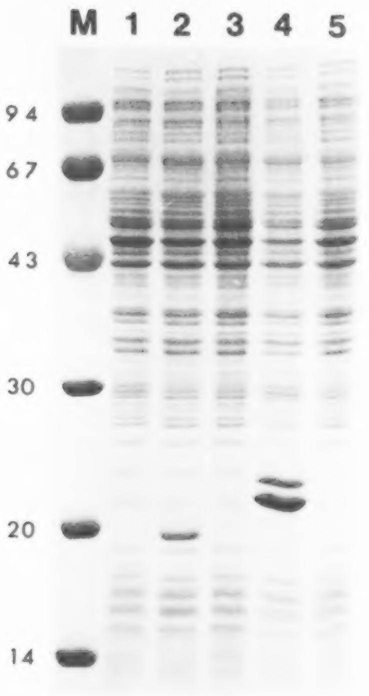
B

```
 5607            -35
 CAAAGGAAGACCTTGAAAAATAACAATTTTTGAC

   -10                +1                 5679
CTAAGATGCTTATACTACTTTAAGGGAGGCCCTTCGTA
```

to 5685 of the 8350-bp DNA sequence (Fig. 2). The transcript was initiated at a thymidine residue 18 nucleotides upstream of the start site and 3 nucleotides upstream of the ribosome binding site for r-protein S13 (Fig. 5). Inspection of the sequence in the vicinity of the transcription start site identified consensus *E. coli*-like hexamers. Sequences showing identity in 5 of 6 (TTGAAA for -35) and 4 of 6 (TAAGAT for -10) positions with proper spacing (17 bp) were observed.

III.1.5 Expression and analysis of recombinant proteins. Recombinants containing fragments of interest from this *C. trachomatis* r-protein gene cluster were constructed by subcloning in the vector pUC18; construction was followed by analysis of their protein profile. The recombinants designated pCTRK1, pCTRK2, pCTJS8 (Gray et al. 1991), and pCTRK3, contain genes which encode r-proteins CtrS8e, CtrL5e, CtrL6e and CtrS5e (Fig. 1), respectively. These were transformed into *E. coli* NM522. The cells were harvested, and extracted proteins were resolved by SDS-PAGE. Figure 6 shows the expression of the CtrL6e and CtrL5e gene products after induction with IPTG. The molecular masses of these proteins as determined by SDS-PAGE were calculated to be 23,000 and 17,000 Da, respectively. Surprisingly, no toxicity of heterologous gene products was observed in *E. coli*. In contrast, cells harboring pCTRK1 and pCTRK3 did not grow well and no additional gene products were visualized when compared with *E. coli* cells harboring the vector alone. It has been reported that alteration of r-protein L6 in mutants of *E. coli* causes resistance to gentamicin, an

FIG. 6. Identification and expression of *C. trachomatis* r-proteins in *E. coli*. A Coomassie blue-stained SDS-PAGE analysis of polypeptides synthesized by the vector pUC18 and constructed plasmids in the host NM522 cells is shown. Lanes 1 to 5 represent pUC18, pCTRK2 encoding CtrL5e, pCTRK1 encoding CtrS8e, pCTJS8 encodinging CtrL6e, and pCTRK3 encoding CtrS5, respectively. Approximately equal amounts of protein were loaded on each lane. Standard molecular weight markers are shown in lane M.


M
1
2
3
4
5
94
67
43
30
20
14

aminoglycoside antibiotic (Buckel et al. 1977). Cells harboring the recombinant plasmid pCTJS8 encoding the 23-kDa CtrL6e were tested for susceptibility to gentamicin. Both the recombinant strain and *E. coli* containing only pUC18 were highly susceptible to gentamicin; the minimal inhibitory concentrations (MICs) for each were less than 0.5 μg/ml.

III.2 Characterization of *C. trachomatis* SecY protein

III.2.1 Analysis of chlamydial *secY* gene product.

The last gene in the *spc* operon is *secY* (Fig. 1) which encodes a protein of 457 amino acids (aa) with high proportion of basic residues (acidic:basic=21:44) and an estimated pI of 10.34. The calculated molecular weight of the chlamydial SecY protein is 50,195 Da (Fig. 2). Analysis of hydropathic profiles of the chlamydial SecY protein by the method of Kyte and Doolittle (1982) with a moving window of 11 residues predicts that the chlamydial *secY* gene product has 10 hydrophobic membrane-spanning regions (designated TMI-TMX). These are interspersed among 6 cytoplasmic (designated CD1-CD6) and 5 periplasmic (designated PD1-PD5) hydrophilic domains (Fig. 7), sharing a similar pattern with the *secY* gene products of *E. coli* (Akiyama and Ito, 1987) and *B. subtilis* (Suh et al. 1990; Nakamura et al. 1990). Amino acid sequence comparison of the chlamydial SecY protein with the *E. coli* and *B. subtilis* SecY proteins showed that the chlamydial SecY protein shares 27.4% and 35.7% identities with the *E. coli* and *B. subtilis* SecY proteins, respectively (Fig. 8). The regions of highest similarity in the primary

FIG. 7. Hydropathy profile of the chlamydial SecY amino acid sequence. The horizontal scale indicates the number of amino acid residues from initiator methionine. The various transmembrane (▬), cytoplasmic (▭), and periplasmic (▨) domains are indicated.

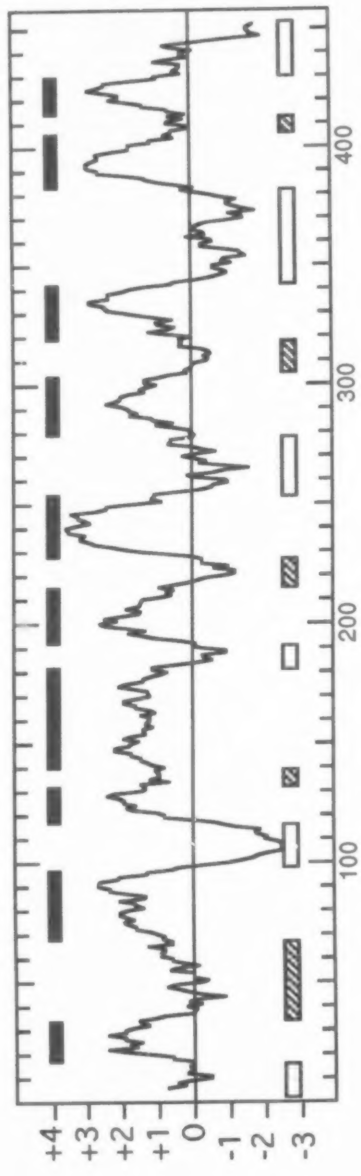

+4
+3
+2
+1
0
-1
-2
-3
100
200
300
400

FIG. 8. Alignment of SecY protein sequences from *C. trachomatis* (CT), *E. coli* (EC) and *B. subtilis* (BS). The single letter amino acid abbreviation are used. Vertical lines indicate identical amino acids. Other abbreviations used are: TM, transmembrane segment; CD, cytoplasmic domain and PD, periplasmic domain. Numbers on the right of each row indicate the positions of amino acid residues.

```
                                TMI
              CD1       ┌───────────────────┐               PD1
ECSECY    MAKQPGLDFQSAKGGLGELKRRLLFVIGALIVFRIGSFIPIPIDAAVLAKLLEQQRGTIIEMFNMFSGGA    70
          ||        |           ||       ||     |      |    ||   ||        |||||
CTSECY    MATLRQVFSISELRQKIFFTFSLL ALCRIGVFIPVPGINGDRAVAYFNQLLGSSRG LFQLADIFSGGA    68
                    |  | || ||    |    ||| |||||             |   |    | |   | |||
BSSECY           MRVSDIRNKIIFTLLML IVFRIGAFIPVP       YVNAEALQAQSQM VFDLLNTFGGGA    54

                     TMII                                    TMIII
          ┌────────────────────────┐        CD2        ┌───────────────┐PD2
ECSECY    LSRASIFALGIMPYISASIIIQLL  TVVHPLAEIKKEGESGRRKISQYTRYGTLVLAIFQS    IGIA    134
                 |||  |||||||| |||         |       | ||    ||  |||||  ||
CTSECY    FAQMTVIALGVVPYISASIIVQLLVVFMPTLQREMRESPDQGKRKLGRMTRLFTLVLACVQSLLFAKFAL    138
            |    | |  ||| |||| |||         |       | ||    || || ||           ||
BSSECY    LYQFSIFAMGITPYITASIIIQLLQMDVVPKFTEWSKQGEVGRRK AQFTRYFTIVL       GFIQAL    116

                           TMIV                                     TMV
          ┌────────────────────────────────────────┐ CD3  ┌──────────────────────
ECSECY    TGLPNMPG      MQGLVINPGFAFY FAVVSLVTGTMFLMWLGEQITERGIGNGISIIIFAGI         192
                ||                 ||   ||   |||  ||| ||||   ||||||| ||  ||
CTSECY    RMNLVVPGIVLPAMLSLKLFGVPWVFYLTTVVVMTTGTLLLMWVGEQISDKGIGNGISLIITLGILASFP    208
                       ||     ||    ||    | | ||  |||  |||   | ||||| ||  ||  | |
BSSECY     GMSYGFNNLANGML IEKSGV  STYLIIALVLTGGTAFLMW LEQITSHGVGNGISIIIFAGIVSSIP    181

                                    TMVI
          ───────┐  PD3   ┌──────────────────────────┐         CD4
ECSECY    VAGLPPAIAHTIEQARQGDLHLVLLLVAVLVF AVTFFVVFVERGQRRIVVNYAKRQQGRRVYAAQSTHL    261
                                | ||    ||  |    |    | | | |  | |  |||        |
CTSECY    SVLGSIFNKLNLGSQDPSEFGIVSLLILCAVFVFVLIATVLIIEGVRKIPVQHARRIIGRREVVGGGSYL    278
             | |      || |     ||    |      |      |   ||||  | |          |    |
BSSECY    KTIGQIYETQFVGSNDQLFIHIV KVALLVIAIAVIVGVIFIQQAVRKIAIQYAKGTGRSPAGGGQSTHL    250

                   TMVII                               TMVIII
          ┌────────────────────────┐   PD4    ┌───────────────────────┐
ECSECY    PLKVNMAGV PAIFASSIILFPATI  ASWFGGGTGWNWLTTISLYLQPGQPLYVLLYASAIIFFCFFYA    328
          ||||| ||| | |||||   |||||               |  |         ||||      |
CTSECY    PLKVNYAGVIPVIFASSLLMFPATIGQFLSSESSWLKRIATMLSPGSVAYSIFYVLLIIFFTYFWTATQF    348
          ||||| |||||||||      | ||           | |              || ||| ||||    |
BSSECY    PLKVNPAGVIPVIFA VAFLIPRTI ASFFGTNDVTKWIQNNFDNTHPVGMAIYVALIIAFTYFYAFVQV    318

                                                  TMIX
                    CD5                ┌───────────────────────┐PD5 ┌───────────
ECSECY    LVFNPRETADNLKKSGAFVPGIRPGEQTAKYIDKVMTRLTLVGALYITFICLIPEFRDAMKVPFYFGGTS    398
                                 |      |     | |  |
CTSECY    RPEQIASEMKKNGAFIPGIRQGKPTQTYLEYTMNRVTLLGAVFLAVVAILPSVLGRILRVDANVSYFLGG    418
           |||      | |  ||| | || ||        | |  |  ||||  |||                  |
BSSECY    NPEQ MADNLKQGGYIPGVRPGKMTQDRITSILYRLTFVGSIFLAVISILP   IFFIQFAGLPQSAQIG    384

          TMX
          ─────────┐          CD6
ECSECY    LLIVVVVIMDFMAQVQTLMMSSQYESALKKANLKGYGR                                    436
               ||        |                    ||
CTSECY    TAMLIVVGVILDTMKQIDAFLLVRRYDGVLKKDRPKGRP                                   457
          |  |||||| | ||||     || |      |
BSSECY    TSLLIVVGVALETMKQ LESQLVKRNYRGFMKN                                         416
```

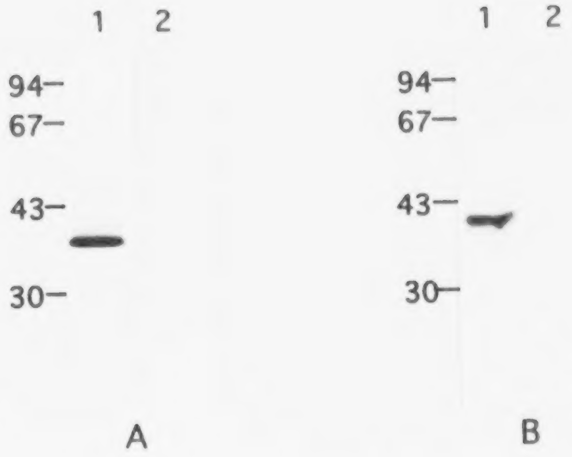
TABLE 2. Comparative analysis of transmembrane segments of SecY proteins from *C. trachomatis*, *E. coli* and *B. subtilis*. The numbers in parentheses indicate the number of amino acid residues in each segment.

segment	amino acids	*E. coli* SecY (% identity)	*B. sub* SecY(%) (% identity)
TMI	17-37 (21)	19.0	57.1
TMII	69-98 (30)	46.7	43.3
TMIII	118-134 (17)	52.9	35.3
TMIV	140-183 (44)	29.5	29.5
TMV	192-217 (26)	34.6	50.0
TMVI	227-255 (29)	31.0	24.1
TMVII	280-306 (27)	66.7	59.3
TMVIII	319-343 (25)	28.0	36.0
TMIX	382-407 (26)	11.5	38.5
TMX	413-429 (17)	11.8	52.9
whole C.tr SecY	1-457	27.4	35.7

sequences were mainly located within the ten presumed transmembrane regions, especially in the second, third, fifth, seventh and tenth segments (greater than 45% identity) (Table 2).

III.2.2 Plasmid-directed protein expression. To study the expression of the chlamydial *secY* gene in *E. coli*, two primers were made. Forward primer began at the first codon of the *secY* gene with an overlapping *Nco*I site (CCA TGG CCA TGG CAT CTA TGC GAC AA) and the universal reverse primer (AAC AGC TAT GAC CAT G) were used to amplify the DNA fragment corresponding to the intact *secY* gene and a part of the gene for S13. The amplified DNA fragment was digested with *Nco*I followed by a fill-in with four dNTPs using the Klenow enzyme to blunt the 5' end. The plasmid pT7-7 was digested with *Bam*HI followed by a similar fill-in to generate blunt ends. The *secY* gene was then subcloned into pT7-7 in frame under the control of the $\phi 10$ promoter. The recombinant plasmid was transformed into *E.coli* strain BL21(DE3). The medium was inoculated with bacterium harboring the recombinant plasmid at 1:100 dilution of an overnight culture. The inoculated medium was grown at 37°C for 2 hours before IPTG was added to a final concentration of 1 mM to induce synthesis of T7 RNA polymerase. 30 min after IPTG induction, 0.2 mM rifampicin was used to stop RNA synthesis by the bacterial RNAP. 30 min after addition of rifampicin, the cells were labeled with [^{35}S]-methionine for 5 min and harvested. Figure 9A shows the expression of *secY* gene product. A protein of 36 kDa was identified on a 10% SDS-PAGE (lane 1). As expected, no SecY specific polypeptide was seen in *E. coli* harboring

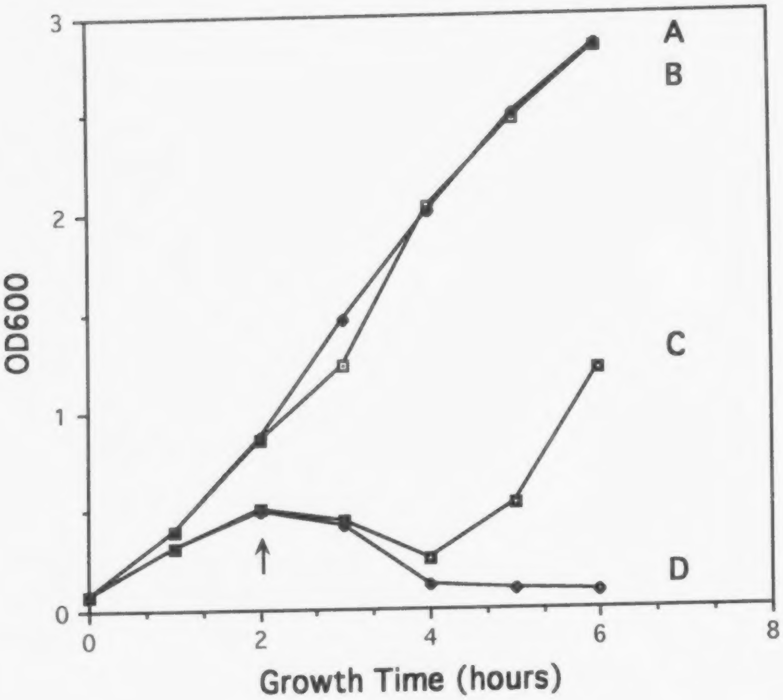
FIG. 9. Expression of *C. trachomatis secY* gene product in *E. coli* BL21. Cells harboring plasmid pT7-7 (lanes 2) or recombinant pCTJHP (lanes 1) were induced with IPTG followed by labeling with [^{35}S]-Methionine for 5 min. Cell extracts in SDS buffer were boiled prior to electrophoresis in 10% (panel A) or 15% (panel B) polyacrylamide gels. Numbers on the left represent molecular weight markers.


1
2
94—
67—
43—
30—
A
1
2
94—
67—
43—
30—
B

pT7-7 alone (lane 2). Surprisingly, the *secY* gene product revealed different molecular weights when analyzed on different gel concentrations. Molecular weight ranges from 36 kDa on 10% SDS-PAGE to 39 kDa on a 15% gel (Fig. 9B lane 1).

III.2.3 Complementation of the *E. coli secY* ts24 mutant with the *C. trachomatis secY* gene product. To define the complementary role of chlamydial SecY in *E. coli*, an experiment was designed to identify whether the chlamydial *secY* gene product is able to complement an *E. coli secY* defective mutant (*E. coli secY* ts24 mutant IQ85) in which the SecY protein is not functional at 42°C (non-permissive temperature). The *Nco*I-*Pst*I DNA fragment containing the intact *secY* gene was subcloned into the prokaryotic expression vector pTrc99A in frame such that the *secY* gene was controlled by the *Trc* promoter in the vector. The recombinant was transformed into *E. coli secY* ts24 mutant IQ85. Cells harboring the plasmid pTrc99A alone were used as a control. The transformed cells were induced with 1 mM IPTG for 2 hours after 1:100 dilution of an overnight culture and the cell growth was monitored at OD_{600}. Figure 10 shows that the cells harboring pTrc99A alone grew in a almost linear manner, and was not affected by IPTG induction. The cells harboring *secY* gene grew slowly compared with control even during the first 2 hours before IPTG induction. The cell density decreased after IPTG induction, suggesting that *secY* gene product is toxic to the host cells. Interestingly, growth of the cells harboring the *secY* gene without IPTG induction decreased during the time between 2 hours and 4 hours but started increasing rapidly after 4

FIG. 10 Complementation of *E. coli secY* ts24 mutant IQ85 with chlamydial *secY* gene. *E. coli* strains carry either vector pTRC99A alone with or without IPTG induction (A and B) or pUCHP containing the *C. trachomatis secY* gene with or without IPTG induction (D and C) growing at 37°C. Arrow indicates the time of IPTG induction.



3
2
1
0
OD600
0
2
4
6
8
Growth Time (hours)
A
B
C
D
↑

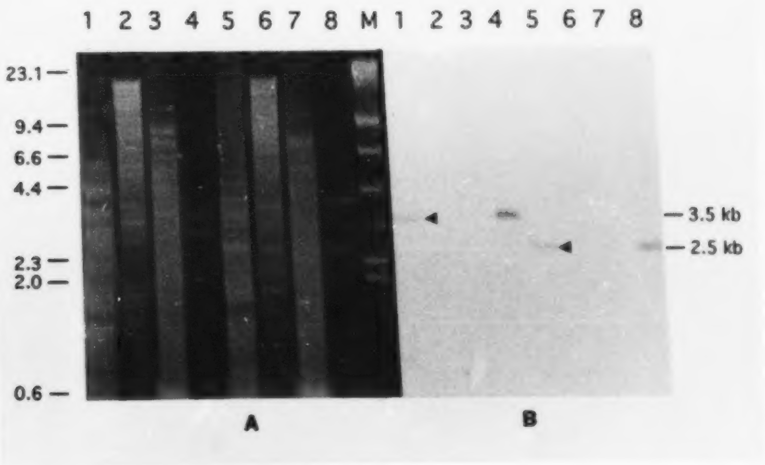
hours. The toxicity of chlamydial *secY* gene product to the *E. coli* host cells made it impossible to perform the complementation of *E. coli secY* mutant with chlamydial *secY* gene.

III.3 Molecular characterization of *C. trachomatis* α r-protein gene operon and RNAP α subunit

III.3.1 Cloning and identification of *C. trachomatis* α r-protein gene operon. Identification of genes downstream of *C. trachomatis* r-protein gene *S13* was undertaken by constructing recombinant libraries of genomic DNA digested with *Bam*HI-*Hin*dIII in plasmid pBluescript SK+. Two colonies containing chlamydial DNA that hybridized to a 906-bp *Bam*HI-*Pst*I fragment of *secY* were used for further studies. The size of these two colonies was very small as compared to other recombinant clones. The recombinant plasmid designated pCTA1 was used for further studies (Fig. 1).

To establish that the cloned gene fragment was chlamydial in origin and that no rearrangements had occurred during cloning, Southern blot analysis was carried out on genomic digests from *C. trachomatis* and pCTA1. Both chlamydial DNA genomic and plasmid pCTA1 revealed a single comigrating band of 3.5-kb when digested with *Bam*HI-*Hin*dIII (Fig. 11; lanes 1 and 4) and a 2.5-kb band when cleaved with *Hin*dIII-*Pst*I (Fig. 11; lanes 5 and 8) upon hybridization with a *Sal*I-*Rsa*I fragment (nt 6438-7368 in Fig. 2) of pCTA1 containing the α gene. The probe DNA was generated by labeling with DIG-dUTP using the random primer method followed by incubation of the Southern blot with Lumi-Phos 530 according to

FIG. 11. Southern blot analysis of the *C. trachomatis* RNAP α subunit gene. Genomic DNA from *C. trachomatis* (lanes 1 and 5), *B. subtilis* (lanes 2 and 6), *E. coli* (lanes 3 and 7) and plasmid pCTA1 (lanes 4 and 8) was digested with *Bam*HI and *Hin*dIII (lanes 1-4) or with *Hin*dIII and *Pst*I (lanes 5-8). After agarose gel electrophoresis and Southern transfer, the DNA was probed with a *Sal*I-*Rsa*I fragment of plasmid pCTA1 containing the chlamydial α gene (Panel B). Panel A represents the ethidium bromide stained gel prior to Southern transfer and hybridization. The numbers on the left show the sizes of *Hin*dIII digested lambda DNA fragment. The actual migration of these markers is shown in lane M. The numbers on the right refer to the sizes of the bands that hybridized to the probe DNA.


1 2 3 4 5 6 7 8 M 1 2 3 4 5 6 7 8
23.1
9.4
6.6
4.4
2.3
2.0
0.6
3.5 kb
2.5 kb
A
B

the manufacturer's recommendations (Boehringer Mannheim). Chemiluminescent signals were detected on X-ray films. Under identical conditions no band could be visualized from genomic digests of either *E. coli* or *B. subtilis,* suggesting the presence of a *Chlamydia* specific single copy gene (Fig. 11).

III.3.2 Analysis of the deduced amino acid sequence in the *C. trachomatis* α operon. Analysis of the potential ORFs in the *C. trachomatis* α r-protein operon revealed that the chlamydial S13, S11, α and L17 proteins comprise 122, 132, 380 and 122 amino acids with derived molecular masses of 13,894, 13,812, 42,265 and 13,968 Da respectively. Of the 122 amino acid residues predicted for S13, over 50% were identical to those of *E. coli* and *B. subtilis* (Fig. 12) while the S11 gene product shows 54% and 59% identities with the *E. coli* and *B. subtilis* S11 gene products respectively. Further downstream of the S11 stop codon is an AUG initiation codon of the *C. trachomatis* RNAP α subunit gene. This ORF encodes a protein of 380 amino acids with 37% and 38% identities with the *E. coli* and *B. subtilis* α subunits respectively (Fig. 12). Based on alignment of conserved amino acid residues among *E. coli, B. subtilis* and chlamydial α subunits, the α subunit can be subdivided into regions I-IV, with region I further divided into subregions Ia and Ib. Table 3 shows a comparison of amino acid identities among different regions of the α subunits. Analysis of the deduced amino acid sequences and comparison of their primary structure revealed conservation of structurally important regions between *C. trachomatis, E. coli* and *B. subtilis.* Besides regions I and IV, which

FIG. 12. Comparisons of the deduced amino acid sequences of the S13, S11, L17 and α genes in the *C. trachomatis* (CT) α operon with those in *E. coli* (EC) and *B. subtilis* (BS). The single letter amino acid code is used. Dots indicate gaps introduced to optimize alignment. Vertical lines indicate identical amino acid residues. Numbers to the right refer to amino acid residues. Regions I-IV and sub regions Ia and Ib of α protein correspond to domains with highest identity.

```
BSS13  MARIAGVDIP RDKRVVISLT YIFGIGRTTA QQVLKEAGVS EDTRVRDLTE EELGKIRDII D.KLKVEGDL
       | || | |||   |   |||| || |||                   |   ||| || |            |||||
CTS13  MPRIIGIDIP AKKKLKISLT YIYGIGPALS KEIIARLQLN PEARAAELTE EEVGRLNALL QSDYVVEGDL
         || || ||   |   | ||  ||| |   | | | |            || |      |         ||||||
ECS13  .ARIAGINIP DHKHAVIALT SIYGVGKTRS KAILAAAGIA EDVKISELSE GQIDTLRDEV A.KFVVEGDL

BSS13  RREVSLNIKR LIEIGTYRGI RHRRGLPVRG QNSKNNARTR KGPRRTVANK KK  121
       || |   ||| || |  |||  |||  ||||| |  | | ||| || | | | | ||
CTS13  RRRVQSDIKR LITIHAYRGQ RHRLSLPVRG QRTKTNSRTR KGKRKTIAGK KK  122
       ||     ||| |     |||  |||  ||||| |||||| ||| || || |
ECS13  RREISMSIKR LMDLGCYRGL RHRRGLPVRG QRTKTNARTR KGPRKPIKK. ..  117


BSS11  ..MAAARKSN TRKRRVKKNI ESGIAHIRST FNNTIVTITD THGNAISWSS AGALGFRGSR KSTPFAAQMA
            | |        | |||  ||  |   | ||||||||||   || ||| | ||  |  ||| ||  |||  |
CTS11  LVKNQAQKRG VKRKQV.KNI PSGVVHVKAT FNNTIVTITD PAGNVISWAS AGKVGYPGSR KSSAFAATVA
            |     || | |     || |  |  ||||||||||   ||   ||  ||  |  ||| ||  ||| ||
ECS11  .....AKAPI RARKRVRKQV SDGVAHIHAS FNNTIVTITD RQGNALGWAT AGGSGFRGSR KSTPFAAQVA

BSS11  AETAAKGSIE HGLKTLEVTV KGPGSGREAA IRALQAAGLE VTAIRDVTPV PHNGCRPPKR RRV  131
       |  |||      |||  ||   || | ||| |  |||   ||  |  ||| ||| ||||||| || |||
CTS11  AQDAAKAAMS SGLKEVEVGL KGTGAGRESA VRALISSGLI VSVIRDETPV PHNGCRPRKR RRV  132
       |   | |     | |  ||   || | ||||   |||   |      | | ||  ||||||| |  |||
ECS11  AERCADAVKE YGIKNLEVMV KGPGPGREST IRALNAAGFR ITNITDVTPI PHNGCRPPKK RRV  128


BSL17  ....MSYRKL GRTSAQRKAM LRDLTTDLII NERIETTETR AKELRSVVEK MITLGKRGDL HARRQAAAYI
                           | |      ||  |||||||    |||||    | |||| |   |  ||| |
CTL17  .......... .........M LANMLKSLIH NERIETTLPK AKELRRHADK MITLAKKNTL AARRLAVGRL
                           |   ||  ||    | | ||||| ||||||      |||||     | ||||  |
ECL17  MRHRKSGRQL NRNSSHRQAM FRNMAGSLVR HEIIKTTLPK AKELRRVVEP LITLAKTDSV ANRRLAFART

BSL17  R......... .....NEVAN EENNQDAL.. QKLFSDIATR YEERQGGYTR IMKLGPRRGD GAPMAIIELV
                                | |      |||   |||    |  |||| | ||  | ||  |   |||
CTL17  MVRYNTLTSK EARQVKAGDL SAYNVDRRVI GKLFDVLATR FSSRNRGYTR ILKLQNRVGD NAQKCIIEFL
                                  |      |||  |  | | ||  |||| |||   | || ||    ||
ECL17  R......... .......... .....DNEIV AKLFNELGPR FASRAGGYTR ILKCGFRAGD NAPMAYIELV

BSL17  .......... .120

CTL17  A......... .122

ECL17  DRSEKAEAAE 127
```

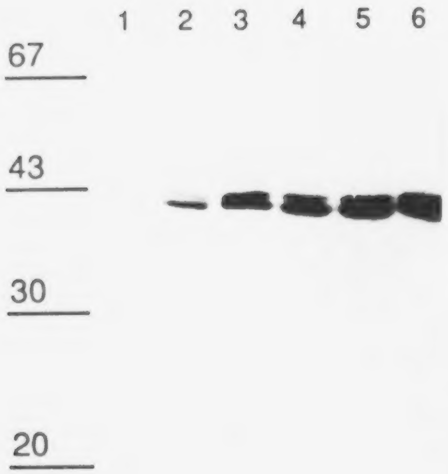
```
                                                 ┌──────────────────────────
                                                 ┌─────── Ia ───────┐
BSALPHA   .......... ....MIEIEK PKIETVEISD DAKFGKFVVE PLERGYGTTL GNSLRRILLS
                           |  |              |   ||    ||| | | || |  ||| ||
CTALPHA   MSDSSHNLLY NKFELPESVK MSPVEGAVGS IDKVARFVAD PLEKGMGHTL GSALRRALLI
                           |  |    |     |     |       ||| | |||| | |||| ||
ECALPHA   .......... MQGSVTEFLK PRLVDIEQVS STH.AKVTLE PLERGFGHTL GNALRRILLS

          ────── I ───────────────────────────┐
                     ┌────────── Ib ──────────┐
BSALPHA   SLPGAAVTSI QIDGVLHEFS TIEGVVEDVT TIILHIKKLA LKIYSDEE.. .........K
           |   |  |     |||||     ||  ||||    |  |    || |
CTALPHA   GLEAPAIVSF SMTGVLHEYM AVEGIIEDVT NMLLNLKGSL LKKYPLQDCE GGRCSQKLRA
               |        ||||||    ||  ||     ||||||
ECALPHA   SMPGCAVTEV EIDGVLHEYS TKEGVQEDIL EILLNLKGLA VRVQGKDE.. .........V

                              ┌───────────────────────────── II ──────────────
BSALPHA   TLEIDVQGEG T......VTA ADITHDSDVE ILNPDLHIAT L.GENASFRV RLTAQRGRGY
          |   |             ||   |       |   ||   | |          |  |    ||||
CTALPHA   TISVDASDLA AAGGQKEVTL GDLLQEGTFE AVNPEHVIFT VT.RPMQLEV MLRVAFGRGY
                |           ||   |    |  |  | | |||    |             |  ||||
ECALPHA   ILTLNKSGIG P......VTA ADITHDGDVE IVKPQHVICH LTDENASISM RIKVQRGRGY

         ─┐                   ┌────────────────────────────── III ───────────
BSALPHA   TPADA...NK RDDQPIGVIP IDSIYTPVSR VSYQVENTRV GQVANYDKLT LDVWTDGSTG
           |                 |   |    ||   | | || |||  ||    | |   | | |||
CTALPHA   SP.SERIVL. .EERGMNEIV LDAAFSPVVL VNYFVEDTRV GQDTDFDRLV LQVETDGRVA
           | | ||      ||         ||  |||     | ||  ||  | || | ||    || |
ECALPHA   VPASTRIHSE EDERPIGRLL VDACYSPVER IAYNVEAARV EQRTDLDKLV IEMETNGTID

          ─────────────────┐
BSALPHA   PKEAIALGSK ILTEHLNIFV GLTDEAQHAE IMVEKEEDQK EKV...LEMT IEELDLSV..
          |||| |     ||  |   |           |      |   |       |    | |  |||
CTALPHA   PKEAVAFATQ ILSKHFSVFE KMDEKRIVFE EAISVEKENK DDILHKLVLG INEIELSVRL
          | ||   |   ||      |        |          ||     | |   |        || |
ECALPHA   PEEAIRRAAT ILAEQLEAFV DLRDVRQPE. ..VKEEKPEF DPI...LLRP VDDLELTV..

          ┌──────────────────────── IV ────────────────────────┐
BSALPHA   .RSYNCLKRA GINTVQELAN KTEEDMMKVR NLGRKSLEEV KAKLEE..LG LGLRKDD...
           || |||  |  | |  ||      |      | | | ||| |  | || |  |  ||
CTALPHA   IRSTNCLSNA NIETIGELVI MPEPRLLQFR NFGKKSLCEI KNKLKEMKLE LGMDLSQFGV
           || |||      |  || ||    |  ||    | ||||| ||  |  |    |  ||| |
ECALPHA   .RSANCLKAE AIHYIGDLVQ RTEVELLKTP NLGKKSLTEI KDVLASRGLS LGMRLENWPP


BSALPHA   .......... .......... ....  314

CTALPHA   GLDNVKEKMK WYAEKIRSSK NTKA  380

ECALPHA   ASIADE.... .......... ....  329
```

TABLE 3. Comparison of amino acid identities among four conserved regions of α proteins between *C. trachomatis* serovar L2, *B. subtilis* and *E. coli*.

Region	% identity between *C. trachomatis* serovar L2 and *B. subtilis*		*E. coli*	
I	50	(29/58)	55	(32/58)
I a	65	(13/20)	75	(15/20)
I b	52	(13/25)	64	(16/25)
I I	34	(15/44)	38	(17/45)
I I I	48	(28/59)	44	(26/59)
I V	48	(25/52)	52	(28/54)

Numbers in parenthesis are ratios of identical amino acids to total numbers of amino acids present in an individual domain.

FIG. 13. Immunoblot analysis of protein lysates from *C. trachomatis* infected host cells during its developmental cycle. Proteins from *C. trachomatis* infected HeLa cells were separated electrophoretically, transferred to a nitrocellulose membrane, and probed with polyclonal antibodies raised to recombinant chlamydial α subunit. Lanes 1-6 represent samples taken at 6, 12, 18, 24, 36 and 48 h post infection respectively. The mobility of low molecular weight markers is shown on the left.

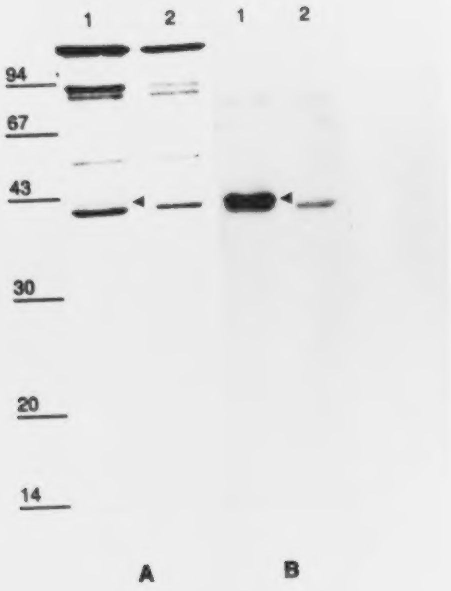

1
2
3
4
5
6
67
43
30
20

represent the amino and carboxyl terminal domains of the chlamydial α subunit protein, significant homology was observed between regions II and III of the central domain.

III.3.3 Development of specific α protein expression in the *C. trachomatis* life cycle. Immunoblot analysis using rabbit anti-chlamydial α antibodies has identified the chlamydial α subunit expression as early as 12 hours post infection. Polyclonal antibodies against the chlamydial α subunit were generated by cloning the *Pst*I-*Hin*dIII fragment of pCTA1 into the vector T7-7 followed by transformation into *E. coli* BL21. Upon induction of these cells with IPTG, a product of 42-kDa was observed on SDS-PAGE. Fig. 13 shows the result of an immunoblot analysis performed on *C. trachomatis* proteins expressed during its growth cycle. Two immunoreactive bands of 42 and 40-kDa were detected as early as 12 hours post infection and persisted to the end of the growth cycle. No bands were detected if HeLa cell lysates were immunoblotted.

III.3.4 Association of the chlamydial α subunit with *E. coli* RNAP. To determine whether the chlamydial α gene product will complement its *E. coli* counterpart, I studied the substitution of the chlamydial α subunit in *vivo*. *E, coli* RNAP was purified from both control and recombinant cells harboring pCTA1. Fig. 14 shows an SDS-PAGE profile of the purified RNAP from control (lane 2) and recombinant cells (lane 1). A lighter band of 42 kDa that moves slower than the *E. coli* α subunit protein was observed in recombinant cell extracts only. Among other common bands was a doublet around 150 kDa which represents the β and β' subunit

FIG. 14. Incorporation of the chlamydial α subunit into *E. coli* RNAP. (A) Coomassie blue stain of *E. coli* RNAP subunits resolved by SDS-PAGE. (B) Immunoblot analysis of the corresponding gel reacted to preadsorbed antichlamydial α polyclonal antibodies. RNAP from cells harbouring pCTA1 (lanes 1) or SK+ (lane 2). The Arrows indicate the mobility of chlamydial α subunit protein. The mobility of low molecular weight markers is shown in kDa on the left-hand side.


1
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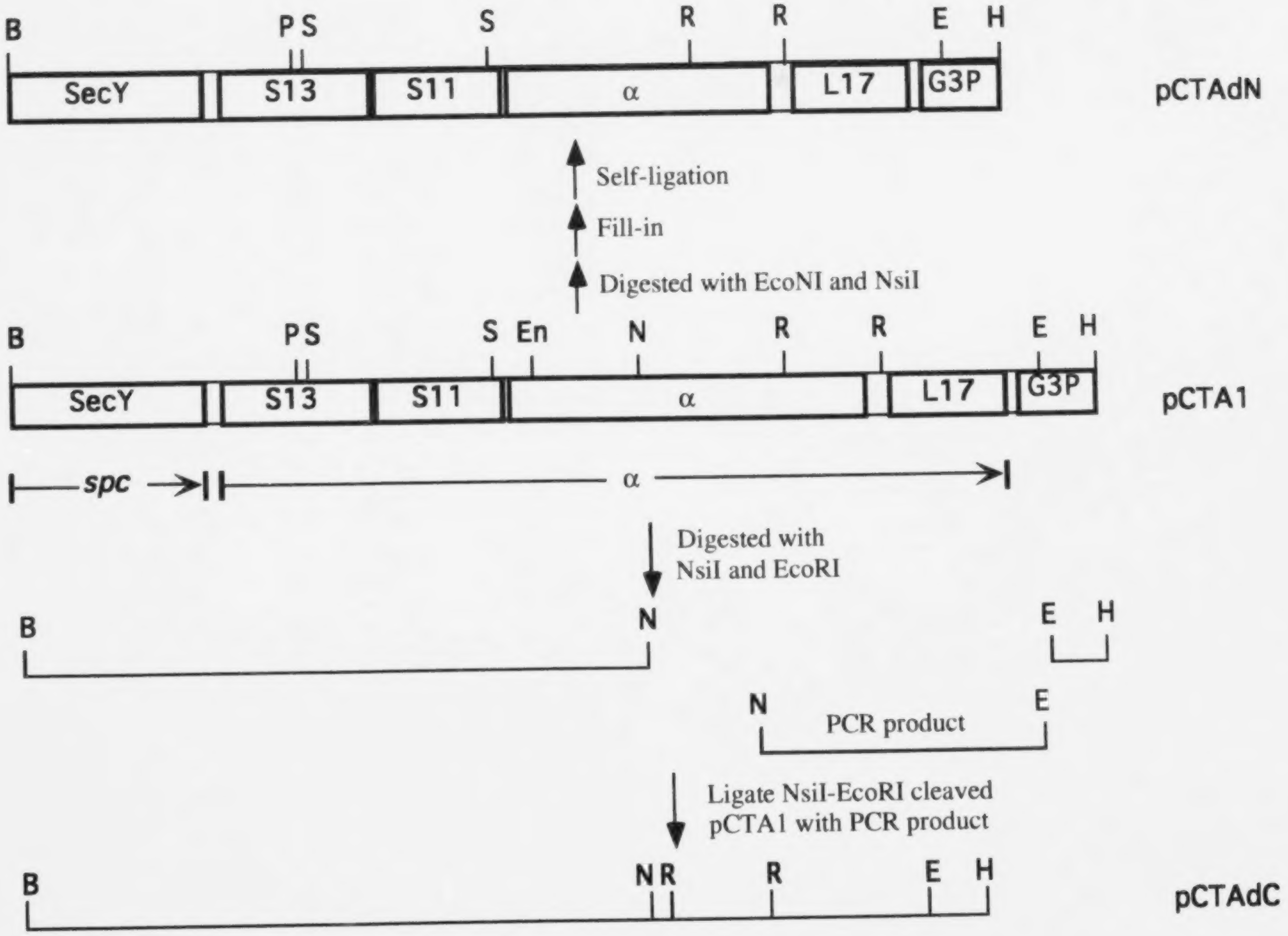
1
2
94
67
43
30
20
14
A
B

proteins. The presence of other bands between the faster moving α subunit protein and the slower moving β and β' subunit doublet may represent different σ factors or their breakdown products. Immunoblot analysis of the corresponding gel revealed that anti-chlamydial α antibodies recognize both *E. coli* and chlamydial antigens with different specificities (Fig. 14). Considering the strength of the Coomassie stained bands, the chlamydial α polypeptide crossreacted more strongly with antibodies than its *E. coli* counterpart (Fig. 14A and 14B; lane 1). These experiments clearly demonstrate the incorporation of the chlamydial α subunit into *E. coli* RNAP.

III.3.5 Construction of chlamydial RNAP α deletion mutants. *In vivo* substitution of the chlamydial α subunit protein into *E. coli* RNAP demonstrated that the chlamydial α subunit protein could incorporate into *E. coli* RNAP. Recent studies have shown that the N-terminus of the α subunit functions in assembly of the RNAP core enzyme while the C-terminus plays an important role in protein-protein contact with some transcriptional factors to increase transcription efficiency. Based on alignment of conserved amino acid residues in the *E. coli*, *B. subtilis* and chlamydial α subunits, the α subunit can be subdivided into regions I-IV. To examine the role of regions I, II and III, the mutants in which these domains were deleted without altering or affecting the flanking cysteine residues were generated. The N-terminus deletion mutant (region I deletion) was constructed by digesting pCTA1 with *Eco*NI and *Nsi*I (nt 6555 and 6861). The resultant product was treated with *E. coli* DNA

Fig. 15. Restriction map of the *C. trachomatis* α operon and its truncated variants. The plasmid pCTA1, encoding the complete α, subunit and its deletion variants, pCTAdN lacking 102 amino acids between positions 25-126 and pCTAdC lacking 137 amino acids between positions 127-264 are shown with the construction strategies. The authenticity of the construct was confirmed by restriction endonuclease digestion and sequence analysis. Abbreviations for the restriction sites are: B, *Bam*HI; E, *Eco*RI; En, *Eco*NI; H, *Hin*dIII; N, *Nsi*I; P, *Pst*I; R, *Rsa*I and S, *Sal*I. Each centimeter in this map represents 200 bp in sequence. The direction of initiation of translation is indicated by arrows.

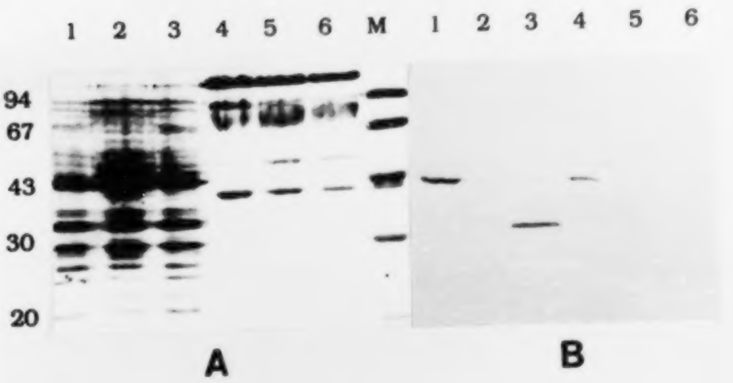

B
P S
S
R
R
E H
SecY
S13
S11
α
L17
G3P
pCTAdN
Self-ligation
Fill-in
Digested with EcoNI and NsiI
B
P S
S En
N
R
R
E H
SecY
S13
S11
α
L17
G3P
pCTA1
spc
α
Digested with
NsiI and EcoRI
B
N
E H
N
E
PCR product
Ligate NsiI-EcoRI cleaved
pCTA1 with PCR product
B
N R
R
E H
pCTAdC

polymerase Klenow fragment in the presence of four dNTPs to generate blunt ends. The blunt ended pCTA1 derivative in which the reading frame of the α subunit gene is maintained was purified by gel electrophoresis and self-ligated. The deletion mutant pCTAdN thus generated lacks amino acids between the position at 25-126 (Fig. 15). The resulting plasmid pCTAdN encodes a polypeptide of molecular mass 30-kDa. The chlamydial RNAP α subunit contains four cysteine residues, two of which viz., Cys^{109} and Cys^{114} are located between regions I and II, while other two residues viz., Cys^{302} and Cys^{334} are located in region IV (Fig. 12). The construction of pCTAdC (central deletion in regions II and III) was designed in such a way that all four Cys residues with possibility of forming intra- and inter-molecular disulfide cross-linking flanked the deleted region. Essentially, two oligonucleotides were synthesized for PCR amplification with forward primer (5' CGA ATG CAT TTG AAG AAG CAA TCT CTG 3') corresponding to nucleotide positions 7279-7297 in which a new *Nsi*I restriction site (underlined) was created. The reverse primer (5' GGG AGA ATT CCT TTT CAG 3') corresponds to nucleotide positions 8179-8162 and carries an internal *Eco*RI site (underlined). Following amplification, the PCR product was digested with appropriate enzymes, gel purified and ligated to *Nsi*I-*Eco*R I linearized pCTA1. The ligated DNA was transformed into competent *E. coli* XL-1 blue cells and positive transformants were confirmed by restriction endonuclease digestion and sequence analysis. A schematic diagram of pCTAdC is shown in Fig 15. Amino acids at the positions from 127 to 264 in region II and region III of the chlamydial α subunit were deleted in the pCTAdC mutant.

III.3.6 *In vivo* association of overexpressed chlamydial α subunit and its deletion variants with *E. coli* RNAP. *In vivo* substitution of the chlamydial α subunit protein and its truncated variant lacking amino acids between the position 25-126 into *E. coli* RNAP was examined. In bacteria, the N-terminal region of α subunit is required for the assembly of the multisubunit RNAP core enzyme. Deletion of the N-terminus from the chlamydial α subunit would prevent it from associating with *E. coli* RNAP. Fig. 16A shows an SDS-PAGE profile of the total cell extracts (lanes 1, 2 and 3) along with the purified RNAPs (lanes 4, 5 and 6) from the *E. coli* cells harboring SK+ (lanes 2 and 5), pCTA1 (lanes 1 and 4) and pCTAdN (lanes 3 and 6). Fig. 16B, showing that the chlamydial α subunit, but not its truncated variant, is incorporated into the *E. coli* RNAP, was obtained by immunoblot analysis. While the strong immunoreactive bands of molecular weight 42 and 30-kDa were observed in the cell extracts from *E. coli* harboring pCTA1 and pCTAdN respectively (Fig. 16B, lanes 1 and 3), no corresponding band was observed in the purified RNAP from *E. coli* harboring pCTAdN (Fig. 16B, lane 6). In contrast, the purified RNAP from the *E. coli* cells harboring pCTA1 revealed a strong immunoreactive band around 42-kDa (Fig. 16B, lane 4) similar in size to the one identified from the corresponding cell extract (Fig. 16B, lane 1). A very weak cross reactivity was observed with *E. coli* α subunit in the purified RNAP (Fig. 16B, lanes 2 and 5).

Some common bands were observed between the purified RNAPs in Fig. 16 irrespective of the plasmids these *E. coli* strains

FIG. 16. Incorporation of truncated chlamydial α subunits into *E. coli* RNAP. (A) Coomassie blue stain of protein in total *E. coli* extracts (lanes 1, 2 and 3) and purified RNAP (lanes 4, 5 and 6) from cells harbouring pCTA1 (lanes 1 and 4), SK+ (lane 2 and 5) and pCTAdN (lanes 3 and 6) following SDS-PAGE. The mobility of low molecular weight markers is shown in lane M while their sizes are represented in kilodaltons on the left-hand side. (B) Immunoblot analysis of the corresponding gel reacted with preadsorbed anti chlamydial α polyclonal antibodies.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harbor. Among other common bands was a doublet around 150 kDa which probably represents β and β' subunit proteins. A very faint band of 42 kDa that moves more slowly than the *E. coli* α subunit is visible for the purified RNAP from *E. coli* harboring pCTA1 only if the proteins are resolved on longer gels. Other bands found in the purified RNAP moving between the faster moving α subunit protein and the slower moving β and β' subunit doublet may represent different σ factors or their breakdown products.

Next, I performed immunoblot analysis on the *E. coli* cells harboring plasmid pCTAdC. This deletion variant lacks the central region of chlamydial α subunit between the amino acids 127 and 264. Total cell extracts from the cells harboring the recombinant plasmids were immunoblotted and probed with the preadsorbed polyclonal antichlamydial RNAP α subunit antibodies. A 27-kDa immunoreactive band was identified in the cell extract harboring plasmid pCTAdC (Fig. 17B; lane 2). The *E. coli* cells harboring plasmids pCTA1 and pCTAdN revealed immunoreactive bands of 42 kDa (Fig. 17B; lane 3) and 30 kDa (Fig. 17B; lane 1) respectively. Surprisingly, The 27 kDa polypeptide of chlamydial α subunit overexpressed in the *E. coli* cells harboring plasmid pCTAdC failed to incorporate into its host RNAP (data not shown)-an observation which suggests that some portion of central region of chlamydial α subunit is involved in the RNAP assembly process.

III.3.7 Purification of overexpressed chlamydial RNAP α subunit and chimeric RNAP from *E. coli*. A significant portion of chlamydial α subunit protein from overexpressing cells

FIG. 17 Expression of the chlamydial RNAP α gene and its truncated variants. (A) Coomassie blue stain of proteins in total *E. coli* extracts from cells harboring pCTAdN (lane 1), CTAdC (lane 2), pCTA1 (lane 3) and SK+ (lane 4) following SDS-PAGE. The mobility of low molecular weight markers in kDa is shown on the left-hand side. (B) Immunoblot analysis of the corresponding gel reacted to preadsorbed antichlamydial α polyclonal antibodies. Arrows show the mobility of overexpressed gene products.

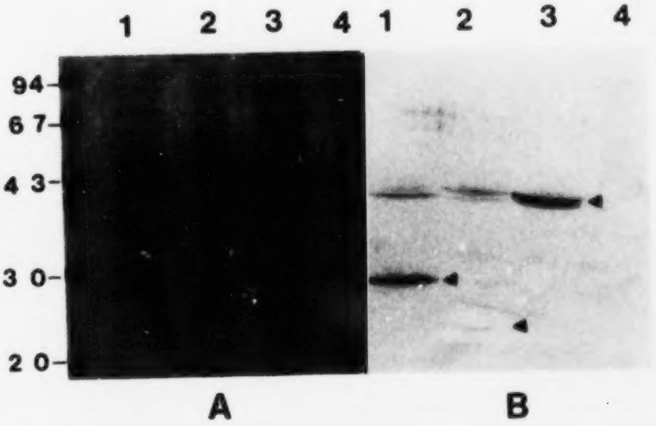
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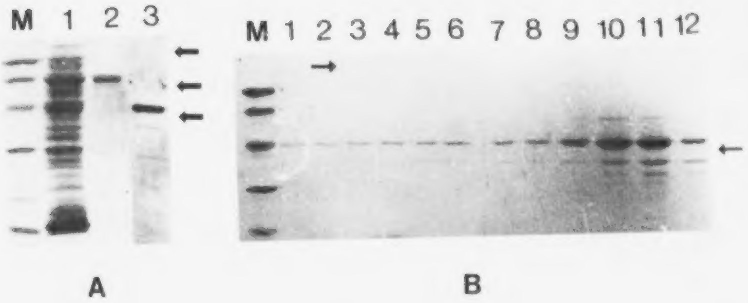
FIG. 18 SDS-polyacrylamide gel showing expression, purification and separation of chlamydial RNAP α subunit. (A) whole cell extracts of recombinant *E. coli* harboring plasmid expressing GST-chlamydial α subunit (lane 1), glutathione-Sepharose affinity purified fusion product (lane 2) and factor Xa cleaved fusion product (lane 3). (B) Factor Xa cleaved product (lane 3) was loaded on a 15-35% glycerol gradient and twelve 0.4 ml fractions were collected (lanes 1-12). The mobility of low molecular weight markers is shown in lane M. Arrows indicate the mobility of E. coli β, β', σ^{70} and chlamydial α subunits in the order of their mobility from top to bottom.


M 1 2 3
M 1 2 3 4 5 6 7 8 9 10 11 12
A
B

was found to be incorporated into *E. coli* RNAP. Whether *in vivo* substitution generates a functionally active enzyme was addressed by isolating *E. coli* RNAP containing heterologous α subunit and assaying it for transcriptional activity. It was reasoned that by generating a fusion protein between chlamydial α subunit and glutathione-S-transferase (GST) followed by overexpression, not only would I affinity purify unincorporated fusion protein but also RNAP associated fusion protein (containing heterologous α subunit) thereby avoiding any contamination arising from homologous *E. coli* α subunit protein. This was achieved by subcloning chlamydial α subunit gene into plasmid pGEX-3X between the *Bam*HI and *Eco*RI sites. These restriction sites were generated by PCR amplification of the RNAP α gene in plasmid pCTA1. The following oligonucleotides were used (forward primer: 5' CCC GGA TCC TGT CGG ATA GTT CAC ACA A 3' and reverse primer: 5' CCC GAA TTC TAG CGT GTT GCA TAT C 3'). Following ligation and transformation, positive clones were identified by colony hybridization and examined for expression of the desired fusion product by SDS-PAGE and immunoblot analysis. Overexpressed fusion product was affinity purified on a glutathione-Sepharose column followed by cleavage with factor Xa according to the manufacturer's recommendation (Pharmacia). Figure 18A shows the overexpressed GST-chlamydial α subunit fusion protein in *E. coli* host strain BNN103 and α subunit protein after digestion of the fusion protein with factor Xa. The α subunit protein purified from the glutathione-Sepharose column contains free α subunit as well as very small amounts of other co-purified proteins which have molecular weights from 24-150 kDa. It was assumed that these co-purified proteins represent *E. coli* RNAP β, β' subunits, σ factors and other transcriptional factors. This assumption was based on my observation that the chlamydial α subunit is incorporated into *E. coli* RNAP to produce chimeric RNAP (Fig. 14). This is because the chimeric RNAP co-purifies with free chlamydial α subunit through its binding to glutathione-Sepharose column.

Dissociated material from the affinity column (comprising both free recombinant α subunit protein and chimeric RNAP) was further subjected to glycerol gradient centrifugation (15-35% glycerol gradient) to separate free recombinant chlamydial α protein from chimeric RNAP as described by Igarashi et al (1991). SDS-PAGE analysis of glycerol fractions confirmed the separation of free from associated complex (Fig. 18B). Trivial amounts of chimeric RNAP can be found in fractions 2 through 4. These fractions reveal the protein bands with different mobilities ranging in size between 42-150 kDa (150 kDa for β, β'; 70 kDa for σ^{70} and 42 kDa for chlamydial α subunit). The chlamydial α protein is predominant in fractions 9 to 11; other protein bands scattered in the fractions may represent other σ factors or breakdown products of RNAP subunits.

III.3.8 Functional analysis of chlamydial RNAP α subunit and chimeric *E. coli* RNAP. Individual fractions containing either free or complexed α subunit were tested for their ability to initiate *in vitro* transcription from a 2.1 kb *Bam*HI-*Kpn*I fragment (Kaul et al. 1990a) encompassing the entire chlamydial MOMP gene with the P1 and P2 promoters as well as upstream and downstream flanking sequences. pUC18 (containing a *lac*Z promoter) was used as a control. Free α subunit (fraction 10) without RNAP failed to transcribe from either MOMP or pUC DNA templates, while chimeric RNAP (fraction 3) initiated transcription from either template but preferentially from the chlamydial MOMP gene, suggestive of its functionality (Fig. 19B and A). In contrast, authentic chlamydial RNAP (high concentration) isolated from six flasks of RB at 18-20 hours post infection initiated transcription from chlamydial MOMP promoter but very little from *E. coli* promoters (Fig. 19C). Interestingly, a significant increase in transcriptional activity was observed for the MOMP transcripts following preincubation of the template DNA with free chlamydial α subunit prior to initiation of transcription by chlamydial RNAP (Fig. 19 D) (Table 4). Although chlamydial RNAP (low concentration) isolated from two flasks of RB-infected HeLa cells inefficiently initiated transcription from either MOMP or pUC genes (Fig. 19 E), preincubation of the template DNA with free chlamydial α subunit significantly increases chlamydial RNAP transcription efficiency (Fig. 19 F). However, some increase in transcription by preincubation of free α subunit was also observed with the pUC template DNA (Fig. 19 D and F).

Recent studies have shown that the C-terminus of RNAP α subunit mediates two functions: protein-protein contact with some transcriptional factors (Ebright, 1993; Thomas and Glass, 1991) to increase transcription efficiency and binding to the DNA UP element of promoter region. To test the ability of the chlamydial α subunit to bind to a DNA promoter region, a 680-bp DNA fragment containing the P1 and P2 promoters for the chlamydial MOMP gene was isolated

FIG. 19 Blot analysis of *in vitro* transcription. *In vitro* transcription assays were performed as described (Mathews et al. 1993) using glycerol gradient fraction 3 containing chimeric *E. coli* RNAP (A); fraction 9 containing free α subunit (B); RB cell free extract with high (C) or low (E) concentration of authentic chlamydial RNAP; and chlamydial cell free extract of different concentration plus glycerol fraction 9 containing purified α subunit (D and F). Plasmid pCT40-118 encoding the chlamydial MOMP gene (Kaul et al. 1990a) served as template DNA in these reactions. ^{32}P-labeled RNA from each reaction was used as a probe. Lanes 1-9 represent 1000, 500, 250, 125, 62.5, 31.2, 15, 7.5 and 3.75 ng respectively of pUC and MOMP DNA blotted onto the membrane.

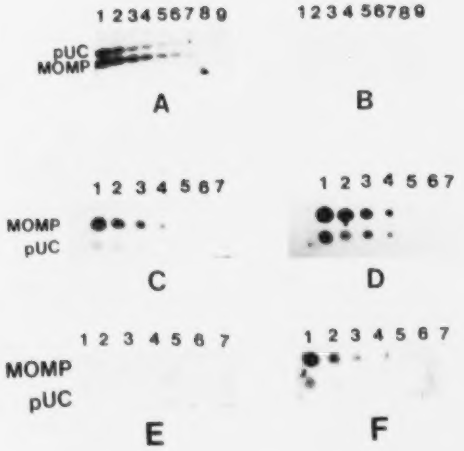
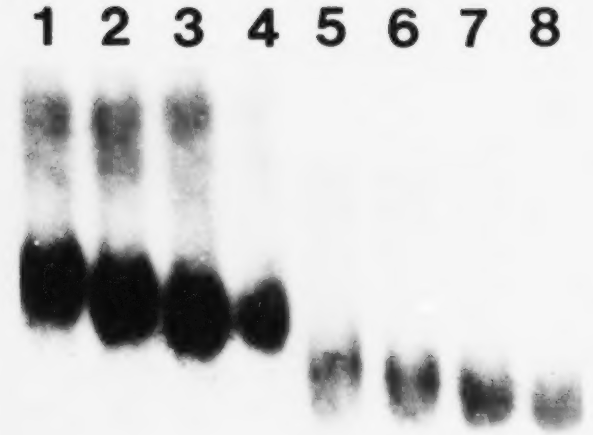

1 2 3 4 5 6 7 8 9
pUC
MOMP
A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7
MOMP
pUC
C
1 2 3 4 5 6 7
D
1 2 3 4 5 6 7
MOMP
pUC
E
1 2 3 4 5 6 7
F

TABLE 4 Results of *in vitro* transcription initiated by chimeric and chlamydial RNAPs.

	Protein Source	Template DNA	
		pUC	Ct MOMP
a)	Chimeric E. coli RNAP	+	+
b)	Chlamydial RNAP	-+	+
c)	Chlamydial RNAP + free recombinant α subunit	+	++
d)	Free recombinant α subunit	-	-

FIG. 20 Mobility shift of the 680-bp DNA fragments containing MOMP P1 and P2 promoter region after incubation with different concentrations of purified free chlamydial α subunit. Each lane represents different ratio of DNA:α subunit molecules from 1:1, 1:0.5, 1:0.25 to 1:0 in lane 1 to lane 4 respectively. The 550-bp DNA fragments containing the MOMP gene (lane 5-8) were used as control at the same concentrations of purified free chlamydial α subunit.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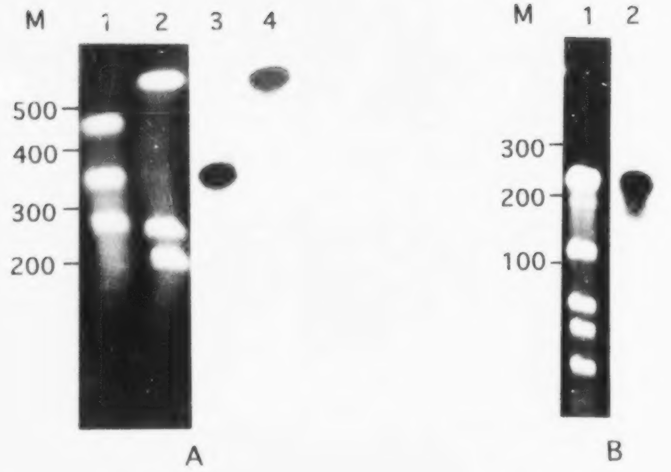
by digesting a 2.1-kb *KpnI-BamHI* fragment with *EcoRI* followed by gel purification. The purified 680-bp DNA fragment labeled with [α-^{32}P]-dATP. The labeled DNA fragment was incubated with different amounts of purified free chlamydial α subunit at room temperature for 30 min followed by electrophoresis on a 4% polyacrylamide gel. Fig. 20 shows the mobility shift of the 680-bp DNA fragments after incubation with different concentrations of the chlamydial α subunit (lanes 1-4). A 550-bp DNA fragment containing MOMP gene was used as a control (lanes 5-8).

III.4 Localization of the chlamydial r-protein *S10-spc-α* gene operons

Agarose-embedded *C. trachomatis* chromosomal DNA was digested overnight with restriction enzymes *NotI* (Fig. 21A lane 1), *SgrAI* (Fig. 21A lane 2) and *Sse8387I* (Fig. 21B lane 1). The products were separated by pulsed field gel electrophoresis with 0.5 X TBE running buffer. The resolved chromosomal DNA fragments were denatured and transferred onto nitrocellulose membranes, followed by hybridization with the 1.7 kb *HindIII-PstI secY* gene fragment as a probe. The result showed that the 1.7 kb *HindIII-PstI* fragment hybridized with the B fragment of the *NotI* digest (Fig. 21A lane 3), the A fragment of the *SgrAI* digest (Fig. 21A lane 4) and the A fragment of the *Sse8387I* digest (Fig. 21B lane 2). Based on the *C. trachomatis* genome map published by Birkelund and Stephens (1992), it is deduced that the chlamydial *S10-spc-α* r-protein gene cluster is located within the region between the genes for the chlamydial RNAP β, β', and σ^{66} subunits (Fig. 22).

FIG. 21 Localization of *S10-spc-α* ribosomal protein gene cluster in the *C. trachomatis* genome. *C. trachomatis* chromosomal DNA was digested with *Not*I (panel A, lane 1), *Sgr*AI (panel A, lane 2) and *Sse*8387I (panel B, lane 1) followed by staining with ethidium bromide. Southern transfer of the resolved chromosomal DNA fragments was followed by hybridization with the 1.7 kb *Hind*III-*Pst*I *secY* gene fragment (panel A, lane 3 and 4, panel B, lane 2). Numbers on the left represent DNA molecular weight markers (kb).


M
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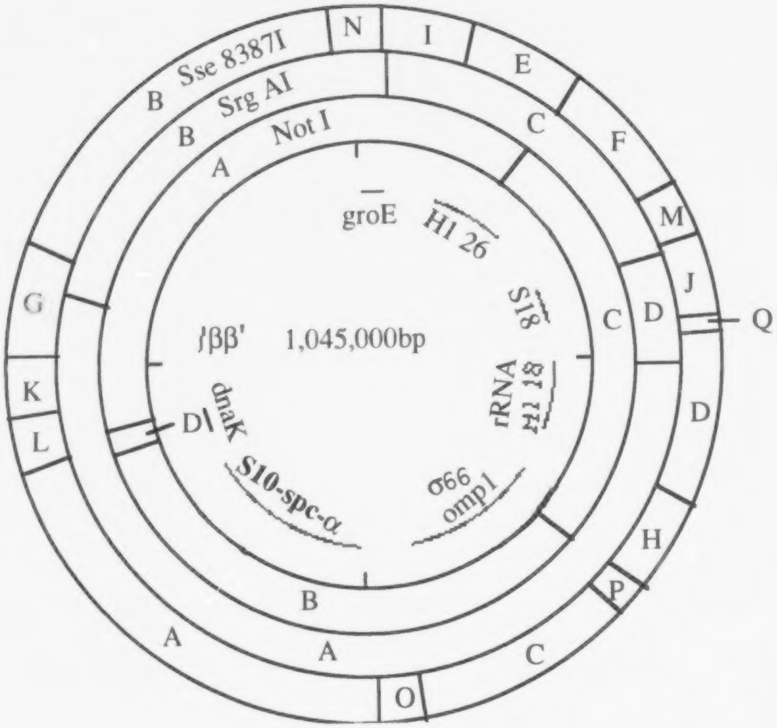
500
400
300
200
A
M
1
2
300
200
100
B

FIG. 22 Circular *C. trachomatis* serovar L2 genome map with the location of chlamydial *S10-spc-α* ribosomal protein gene operons. The arcs in the center indicate the areas in which the genes are localized in the context of the restriction endonucleases *Not*I, *Sgr*I and *Sse*8387I. 1° =2,900 bp


Sse 8387I
Srg AI
Not I
N
I
E
F
M
J
Q
D
H
P
C
O
A
B
G
K
L
D
C
D
C
B
A
B
A
groE
H1 26
18S
rRNA
H1 18
σ66
omp1
S10-spc-α
dnaK
ββ'
1,045,000bp

IV. Discussion

IV.1 Cloning and sequence analysis of *C. trachomatis S10-spc-α* r-protein gene operons

I have characterized a 8350-bp region of the *C. trachomatis* genome that is comparable to the region of the *E. coli S10-spc-α* r-protein gene operons (Fig. 1). The *C. trachomatis spc-α* operons contain the r-protein genes for CtrL14e, CtrL24e, CtrL5e, CtrS8e, CtrL6e, CtrL18e, CtrS5e, CtrL15e, CtrS13e, CtrS11e, CtrL17e and the genes for CtrSecYe and the chlamydial RNAP α subunit, along with the adjacent genes for r-proteins CtrL16e, CtrL29e and CtrS17e. In the 5' region of this gene cluster are similar to the last three genes of *E. coli S10* operon preceding the *spc* operon (Zurawski and Zurawski, 1985). This similarity suggests that the basic organization of the gene cluster was established before the divergence of *C. trachomatis* and *E. coli* in evolution. This hypothesis is consistent with the presence of two overlapping translational stop and start codons in *C. trachomatis* compared with three overlapping regions in *E. coli* (Zurawski and Zurawski, 1985) and four in the *M. capricolum spc* r-protein gene cluster (Ohkubo et al. 1987). Surprisingly, there are no overlapping stop or start codons in the archaebacterium *M. vannielii* despite strong conservation among *spc* r-protein gene cluster with respect to gene comparison and organization (Auer et al. 1989). In Chlamydiae, however, the genes for CtrS14e and CtrL30e are absent from *spc* gene cluster. Whether these two genes are located in a separate region of the chromosome or were lost completely during evolution is not clear from this study. On the basis of the evidence that EcoS14 and *B. stearothermophilus* BstS14e are essential for *in vitro* reconstitution of functionally active 30S subunit (Higo et al. 1973), it is tempting to anticipate that the CtrS14e gene is located distantly in the chromosome. However, the gene homolog for EcoL30 is also absent in *M. capricolum* (Ohkubo et al. 1987). There is 75% bias toward A and U in position 3 of codons in the cluster. This codon usage pattern is similar to one reported earlier from our laboratory and is different from those in *E. coli* (49%) and *M. capricolum* (91%) (Gray et al. 1991). The overall calculated A+T contents of the genes in the cluster among *C. trachomatis*, *E. coli* and *M. capricolum* are 60%, 48% and 69%, respectively (Kaul et al. 1987; Muto et al. 1984). These percentages are consistent with the averages for the whole genomes.

In *E. coli* there is a 170 bp intergenic region between EcoS17, the last gene of *S10* operon, and EcoL14, the first gene of the *spc* operon (Cerretti et al. 1983). This space contains rho-independent termination signals for the upstream *S10* operon as well as promoters and initiation signals for the downstream *spc* operon (Lindahl et al. 1990). In contrast, *C. trachomatis* contains only a 16-bp spacer between the two operons (Fig. 2). No transcription signals were found in this intergenic region, suggesting that the transcription of the *spc* region may initiate from an upstream promoter. Fusion of the *S10* and *spc* operons into a single operon has been reported to occur in *M. capricolum*, which contains a 15-bp spacer. Transcription in *T. aquaticus* and archaebacterium *M. vannielii* as well as

Halobacterium marismortui begins at the *S10* operon and most likely traverses into the *spc* operon (Arndt et al. 1990; Auer et al. 1989; Jahn et al. 1991). Northern blot analysis was used to evaluate whether chlamydial *spc* and *S10* operons are transcribed as single or separate transcriptional units. While an approximate 5-kb transcript was hybridized to the probe from the *spc* operon genes, a different transcript size was obtained when an identical blot was hybridized to *S10* operon genes, evidence for independent transcription of the *spc* and *S10* regions. Further support for the presence of an internal promoter regulating the *spc* operon was obtained by analysis of the transcription initiation site using the primer extension technique (Fig. 4). The apparent promoter shows some similarities to the *E. coli* consensus promoter sequence recognizing the σ^{70} subunit of RNAP (Raibaud and Schwartz, 1984). The -10 sequence shows identity among 5 of 6 positions, while the -35 reveals identity among 2 of 6 positions and includes a highly conserved TTG sequence at its 5' end rather than the conventional 3' end of the -35 region. The lack of homology among the various promoter regions in chlamydiae has made it difficult to define a consensus promoter-like sequence in this organism and also accounts for its failure to initiate any efficient transcription in *E. coli* (Sardinia et al. 1989; Stephens et al. 1988).

The analysis of r-proteins in prokaryotes is tightly coordinated and stoichiometrically balanced with the assembly of mature ribosomes (Lindahl and Zengel, 1979; 1986; Nomura et al. 1984; Zurawski and Zurawski, 1985). Such a coordinated regulation is mediated by the autogenous feedback mechanism whereby certain r-proteins, when unbound by rRNA, prevent the translation of their own mRNA (Gausing, 1980; Yates et al. 1980). However, the mechanism of transcriptional regulation differs among *E. coli* r-proteins (Lindahl et al. 1990; Lindahl and Zengel, 1979; 1986). Recently, one group reported the regulation of *spc* operon genes by translational coupling in which the product of EcoS8 gene binds to the *spc* operon mRNA near the beginning of EcoL5, leading to repression of EcoL5 translation and subsequently the downstream *spc* operon (Mattheakis et al. 1989). The same group (Henkin et al. 1989), however, failed to observe similar structures in the *B. subtilis* gene cluster, prompting them to speculate that the translational feedback regulation system for control of r-protein gene expression is fairly recent in evolution. Computer searches in and around the CtrL5e mRNA sequence failed to identify the structure similar to the EcoS8 target site. These studies suggest the existence of an alternate pathway for *spc* operon autoregulation in Chlamydiae. Similar observations have been made for *T. aquaticus* (Jahn et al. 1991). These results indicate a high degree of conservation among r-protein structure and organization despite the evolutionary distance between *E. coli* and *C. trachomatis* (Moulder, 1988; Palmer et al. 1986; Weisburg et al. 1986).

Overproduction of some r-proteins in *E. coli* can lead to host toxicity or cell death because of the imbalance in stoichiometry of ribosome synthesis or autogenous regulation. As discussed, the endogenous r-protein genes are subject to feedback inhibition to minimize any reduction in ribosome activity. Recently, it was reported from our laboratory that CtrL6e substituted into *E. coli* ribosomes without any deleterious effect despite its overproduction (Gray et al. 1991). To study the effect of other chlamydial r-protein gene products on *E. coli* growth and survival, *E. coli* cells were transformed with plasmids containing genes for CtrL5e, CtrL6e, CtrS5e and CtrS8e. Only the gene products from CtrL5e and CtrL6e were visible *in vitro* when resolved by SDS-PAGE (Fig. 6), suggesting an unstable nature of the CtrS5e and CtrS8e transcripts and/or their gene products in *E. coli*. *B. stearothermophilus* BstS5e also appears to be toxic to *E. coli* in spite of the fact that endogenous overproduction of EcoS5 has not led to any toxicity (Nomura et al. 1984). The product of CtrS8e could be lethal by virtue of its characteristic binding to the translational repressor in *E. coli*.

Unlike many gram-negative bacteria, chlamydiae are completely resistant to the aminoglycoside gentamicin. In *E. coli*, alterations in EcoL6 have been reported to cause gentamicin resistance, invoking it as a possible target site (Buckel et al. 1977). These studies prompted me to look for such a situation with CtrL6e, the chlamydial EcoL6 homolog. *E. coli* harboring the recombinant pCTJS8 encoding CtrL6e were highly susceptible to gentamicin, suggesting that the intrinsic aminoglycoside-resistant locus in *C. trachomatis* is located at some other place in the genome. Alternatively, the resistance could be ascribed simply to the impermeability of EBs to aminoglycosides because of their highly disulfide-linked outer membrane and to a protective intracellular host environment in which RBs are located.

IV.2 Biochemical and genetic characterization of the chlamydial *secY* gene and its gene product

The cell wall of *Chlamydia* contains both inner and outer membranes, but unlike other gram negative bacteria, it lacks demonstrable peptidoglycans. Recent studies have clearly shown the existence of protein processing machinery in *Chlamydia* (Kaul et al. 1990a). However, the extent of its secretory apparatus remains unelucidated. In *E. coli*, the process of protein translocation and secretion appears complex and well orchestrated (Ito, 1992). Central to the process of protein translocation is the role of the *secY* gene product which acts as a translocator through which proteins cross the inner membranes (Bieker and Silhavy, 1990; Bieker et al. 1990; Brundage et al. 1990). To define the genetic conservation and structure-function relationship of this essential secretory component, a 1.7-kb *Hin*dIII-*Pst*I fragment, designated pCTJHP, encoding the entire chlamydial SecY was cloned and sequenced (Fig. 1 and Fig. 2). The deduced nucleotide sequence comprises three ORFs homologous to *E. coli* L15, SecY and S13 of *spc-α* r-protein operons. The similarities in the basic organization of this gene cluster among various gram negative and gram positive organisms points out its functional importance and provides evidence for its existence before evolutionary divergence. In *Chlamydia*, however, the gene for protein X is missing. The X gene, located between *secY* and *S13* in *E. coli*, appears essential for bacterial growth and protein export (Ito, 1990). Recently it has been reported that the X gene encodes a new large subunit r-protein (L36), thus termed *rpmJ* (Wada and Sako, 1987; Ueguchi et al. 1989). Further studies of the function of the X (L36) gene product suggested that an intact *rpmJ* region is important in maintaining normal levels of *secY* gene product and seems not to be essential for protein export and bacterial cell growth (Ueguchi, 1989). Whether *Chlamydia* has lost this gene during evolution or it exists elsewhere on the chromosome is not evident from this study.

The CtrSecY homolog from *Chlamydia* contains 10 probable hydrophobic transmembrane (TM) segments, interspersed by 11 hydrophilic domains comprising 6 cytoplasmic domains (CD) and 5 periplasmic domains (PD) (Fig. 7 and Fig. 8). It shares similar topologies to that of the SecY proteins from *E. coli* (Akiyama and Ito, 1987), *B. subtilis* (Nakamura et al. 1990; Suh et al. 1990), some archaebacterium (Arndt, 1992; Auer et al. 1991) and the plastid genome of the alga *Cryptomonas* ϕ (Douglas, 1992). Although amino acid sequence homology among *C. trachomatis*, *E. coli* and *B. subtilis* proteins is not very high, more than 45% homology exists among various TM regions (Table 2), suggesting that these regions are essential components of a protein translocation tunnel. Changes in TM segments that contain conserved residues affect both protein function and signal sequence recognition. It is not surprising, therefore, that TM segment VII, which is highly conserved among most SecY proteins including *Chlamydia*, has been invoked in signal sequence recognition. Two out of four *prlA* signal sequence mutations, along with SecY121 and SecY161 mutations, have been mapped to this region in *E. coli* (Sako and Iino, 1988; Sako, 1991). In spite of evolutionary distance, eubacteria and some primitive plants share the basic structure of central components for protein translocation across cytoplasmic membranes, suggesting that protein export apparatus existed before evolutionary divergence.

The apparent lack of conservation of the N- and C- terminal cytoplasmic domains (domains CD1 and CD6) suggests that these regions may not play an important role in signal peptide interaction. Similar conclusions have been drawn for the *B. subtilis* SecY protein (Suh et al. 1990). These observations are contrary to the results of Watanabe and Blobel (1989) who proposed a role for terminal domains in signal peptide interaction. Among other cytoplasmic domains, only domains 3 and 5 demonstrated significant homology to the *E. coli* homolog. A temperature sensitive mutation (*secY* ts24), defective in protein export due to amino acid alteration from Gly^{240} to Asp, has been mapped to CD4 (Shiba et al. 1984). This domain also contains the OmpT cleavage site, invoked in EcoSecY proteolysis (Akiyama and Ito, 1987). However, no mutation has been mapped to CD3 in *E. coli* and its functional significance remains unclear.

In *Chlamydia*, four out of five periplasmic domains from PD2 to PD5 bear no homology to *E. coli* SecY. Whether this has any relationship to the absence of peptidoglycans in the chlamydial periplasmic layer is unknown. Only PD1 located between TM1 and TMII shows significant similarity to its *B. subtilis* and *E. coli* homologs. This domain is thought to form the extra membrane loop of EcoSecY and is likely to perform an important EcoSecY function (Ito, 1992).

Biochemical analysis of the *C. trachomatis* SecY protein revealed some unusual characteristics. The observed molecular weight, 36,000 daltons, of the chlamydial *secY* gene product on 10% SDS polyacrylamide gels was much lower than the calculated molecular weight of 50,149 daltons and may reflect the highly basic nature (pI 10.43) of the protein molecule (Fig. 9A). In addition, the hydrophobic nature of CtrSecY protein may necessitate excessive binding of SDS leading to its migrational variability in SDS polyacrylamide gels of varying concentrations (Fig. 9B). A similar observation has been made previously with proteins that are located in cytoplasmic membranes (Ito, 1984; Larson et al. 1982; Stoker et al. 1983; Teather et al. 1978). These characteristics, along with the observation that overproduced protein is degraded quickly over extended periods, closely mimic those of the *E. coli* SecY protein. Similar properties of SecY proteins from different species such as *E. coli* and *C. trachomatis* reflect the relationship between conformational structures and functional characteristics of proteins.

Further studies of the *E. coli* SecY relying on the NH_2-terminal sequence analysis confirmed that the SecY protein is translated from its assigned initiation site and does not undergo N-terminal processing (Akiyama and Ito, 1986) although it contains a potential leader peptidase cleavage site located near the proposed interface between transmembrane segment 5 and periplasmic domain 3 (Akiyama et al. 1990). Recently, Akiyama and Ito (1990) reported that *E. coli* SecY can be specifically cleaved at the central region of the polypeptide (residues 239-270) after cell disruption by OmpT protease (protease VII) which recognizes paired basic amino acid residues of the peptide (Sugimura and Higashi, 1988). Whether protease VII can explain the lower molecular weight of the *C. trachomatis secY* gene product remains to be seen. Interestingly, computer searches identified several stretches of two or three consecutive basic amino acids in the proposed cytoplasmic domain 4 of *C. trachomatis* SecY protein, along with the site Lys^{111}-Arg-Lys in its CD2 region which appears conserved between *E. coli* and *B. subtilis*.

Complementation of function-defective mutants is useful in understanding the mechanism of structure-function relationships and in tracing evolutionary divergence. Complementation studies of an *E. coli secY* mutant (*secY* ts24) with a *B. subtilis secY* homolog are contradictory and controversial (Nakamura et al. 1990; Suh et al. 1990). However, the complementation results of an *E. coli* protein transport defective mutant with the chlamydial SecY homolog revealed that *C. trachomatis* SecY protein is highly toxic to the host cells. The cell density decreased rapidly upon IPTG induction (Fig. 10, D), suggesting that the cells were dying. Overexpressed chlamydial SecY, which is highly hydrophobic, probably integrates into the host cell membrane to cause alteration of the membrane permeability or to block the normal host protein translocation apparatus. In contrast, IPTG induction had no effect on growth of the cells harboring the pTrc99A (Fig. 10, A and B). Interestingly, the cells harboring a plasmid containing *secY* without IPTG induction gave a unique growth pattern (Fig. 10, C) in which the cell growth increased from 0 to 2 hours, decreased from 2 to 4 hours and increased again after 4 hours of growth. In the first 2 growth hours, the cells harboring the plasmid containing *secY* gene (C and D) grew slower compared with the cells harboring vector alone (A and B), which could be explained by a low level of *secY* gene expression and toxicity of *secY* gene product. During the growth time of 2 to 4 hour, cell growth decrease probably reflects effects of accumulation of *secY* gene product from basal leakage on cell growth. After 4 hours of growth, the bacteria are probably able to produce some protease to degrade the *secY* gene product and the cell growth increases rapidly. These results demonstrate that chlamydial *secY* gene product, even with basal leakage from vector, interferes with host cell growth, suggestive of its toxicity to host cells. However, *E. coli* SecY variants have also been shown to interfere with normal *E. coli* SecY protein function and host cell growth (Shimoike et al 1992).

Although we have no direct evidence that the chlamydial SecY homolog is essential for secretion, it is clearly related to *E. coli* SecY by the criteria of high primary sequence similarity, similar hydropathic profile, and similar biochemical characteristics. Furthermore, the *C. trachomatis secY* homolog lies at the promoter-distal end of the *spc* r-protein operon, which is the same relative position occupied by *E. coli secY*. This position may serve an important regulatory role in balancing the secretory and translational capacities of the cell, Interestingly, the fact that the *E. coli secY* gene is located within a r-protein gene cluster is believed to be "the case of the misplaced genes" (Bjork, 1985). Although the *E.*

coli SecY protein has been shown to act post-translationally to promote bacterial protein export (Bacallao et al. 1986) and the level of *E. coli secY* gene expression appears to be about 1/5 the r-protein levels (Ueguchi et al. 1989; Ito, 1990), the relationship between products of the *secY* gene and other r-proteins is still largely unelucidated. Ribosomal proteins constitute ribosomes which are the machinery for protein synthesis while the SecY protein forms a membrane channel for protein export. The location of *secY* among the genes for r-proteins may reflect a harmonious gene regulation mechanism for protein synthesis and translocation.

IV.3 Molecular characterization of the gene for *C. trachomatis* RNAP α subunit

Initial attempts to clone the gene for α subunit of chlamydial RNAP using a PCR based approach, with degenerate oligonucleotide primers made to conserved regions of *E. coli* and *B. subtilis* α subunits, were unsuccessful, as was the approach of using the entire *B. subtilis* α gene as a probe. Subsequently, I took advantage of the fact that in *E. coli*, the α operon is present directly downstream of the *spc* operon. It was assumed that the order of these two operons might be conserved in *Chlamydia* and therefore it should be straight forward to clone following our earlier strategy. By using a DNA walking and subcloning strategy, an overlapping 3.4-kb DNA fragment of *C. trachomatis* was cloned that contained the genes for the α subunit of RNAP and the adjacent genes for CtrS13, CtrS11 and CtrL17. While this work was in progress, the cloning and sequence analysis of the α subunit gene from the *C. trachomatis* mouse pneumonitis biovar (MoPn) was reported (Tan et al. 1993); this has, however, since been found to be in error. The sequence reported applies to *Mycoplasma* spp. rather than *Chlamydia* spp. because of PCR contamination. A Southern blot was performed to ensure that the 3.4-kb *Bam*HI-*Hin*dIII fragment was authentic. Figure 11 demonstrates that the *Sal*I-*Rsa*I fragment of plasmid pCTA1 containing the chlamydial α gene only hybridizes with a 3.5 kb fragment of the genomic DNA digested with *Bam*HI and *Hin*dIII from *C. trachomatis*, strongly indicating that this 3.4-kb *Bam*HI-*Hin*dIII fragment is cloned from the *C. trachomatis* chromosome rather than other sources.

The order of gene organization in this region is similar though not identical to that found in *E. coli* (Bedwell et al. 1985). This order, S13, S11, α and L17 is, however, identical between *C. trachomatis* and *B. subtilis* (Boylan et al. 1989). Also, among plant chloroplast, *B. subtilis* and *C. trachomatis* genomes, the α subunit is always preceded by the *S11* gene (Hiratsuka et al. 1989; Purton and Gray, 1989; Sijben-Muller et al. 1986). However, in the chlamydial α operon, the Ctr*S4* gene is missing. In *E. coli* the gene for S4 separates the genes for S11 and α (Bedwell et al. 1985). This gene product has been invoked in the translational regulation of the *E. coli* α operon. The lack of S4 in the chlamydial α gene operon may suggest an alternative pathway for translational control. These observations are consistent with our studies which failed to identify any r-protein CtrS8 sites in and around the r-protein CtrL5 coding sequence. In *E. coli*, regulation of the *spc* r-protein operon by translational coupling is mediated through the binding of the S8 gene product to the *spc* operon mRNA near the beginning of L5 (Mattheakis and Nomura, 1988). The *S4* gene product binds to its target site within the 100 bp leader sequence of the α mRNA to negatively regulate the gene expression of *E. coli* α operon. Interestingly in *B. subtilis*, the *rps*D gene encoding ribosomal protein BstS4 is monocistronic and located at position 263 degrees on the *B. subtilis* chromosome, outside of the α operon (Grundy and Henkin, 1990; 1991). The leader region of *rps*D mRNA has extensive secondary structure resembling a region of *B. subtilis* 16S rRNA where S4 is likely to bind and the *rps*D gene is autogenously regulated (Grundy and Henkin, 1990). Whether the chlamydial CtrS4 gene has been lost during evolution or is located elsewhere on the chromosome (as is the case for *B. subtilis*) and how the expression of chlamydial α operon genes is regulated remain to be elucidated. Considering that r-protein S4 functions as an auto feedback regulator for α operon gene expression and as a component, along with S1, S5 and S7, comprising the ribosomal mRNA-binding site (Dontsova et al. 1992), the gene for chlamydial S4 r-protein is likely located elsewhere on the chromosome.

Except for CtrS11, all other genes in the chlamydial α operon cluster initiate from ATG and terminate with either TAA or TAG termination codons. No preferential bias towards any specific termination codon was observed. Preceding the initiation codons for the chlamydial CtrS13, CtrS11 and Ctrα genes is a strong Shine-Dalgarno sequence, while no identifiable ribosome binding sites were found upstream of CtrL17. Sequence analysis of CtrS11 gene revealed an unusual UUG initiation codon. This feature is quite rare among genes derived from *E. coli* (Mackie, 1981; Poulis et al. 1981; Kusters et al. 1989; Reddy et al. 1985; Roy et al. 1983), although several genes containing UUG initiation codon have been reported among gram positive bacteria of the *Bacillus* and *Staphylococcus* genera (Kitazono et al. 1992; McLaughlin et al. 1981). Initiation from UUG is believed to decrease the efficiency of translation while its replacement by AUG results in increased expression levels (Reddy et al. 1985). In addition, omission of S11 from the 30S ribosomal proteins of *E. coli* has been shown to decrease the translational fidelity *in vitro* (Nomura et al. 1969). Taken together, these results suggest a possible translational control mechanism for the expression of the chlamydial r-protein CtrS11.

In *E. coli*, the α operon transcript has been reported to originate from a strong *spc* promoter as well as from a promoter that precedes the α operon (Lindahl et al. 1990). Except for a 55-bp intercistronic region between Ctr*secY* and Ctr*S13* genes and a 63-bp region between *α* and Ctr*L17*, the chlamydial α operon genes are closely linked. In *Chlamydia*, evidence for the presence of a potential promoter regulating the chlamydial α operon was obtained by analysis of its transcription start site using the primer extension technique (Fig. 5). Although we observed consensus *E. coli*-like hexamers with proper spacing between -10 and -35 regions upstream of the transcription start site, a -10 promoter sequence showing identity among 4 of 6 position was identified which exhibits a 13-bp spacing from its start site. Whether it represents a true chlamydial promoter or not remains speculative. Further, sequence distal to chlamydial α ORF within Ctr*L17* ORF revealed a 7-bp dyad with the potential of forming a stem and loop structure followed by five uridine residues, indicative of a rho independent terminator for chlamydial α operon (Platt, 1986). Northern blot analysis was used to evaluate the validity of these observations. Unfortunately, no transcript was visualized, probably due to the low level expression of the α operon.

Analysis of the deduced amino acid sequence of the α subunits shows that homologous regions between *E. coli* and *B. subtilis* are essentially conserved in *C. trachomatis* serovar L_2 as well (Fig. 12). Considering the minimal evolutionary relatedness among *C. trachomatis, E. coli* and *B. subtilis* based on 16S rRNA relatedness (Weisburg et al. 1986), the amount of amino acid conservation is significant. The α subunit has been assigned a role mainly in the assembly of the multisubunit complex, serving as a scaffold upon which the rest of the complex is built. Recent evidence suggests that the α protein is also involved in specific promoter sequence recognition of an AT-rich UP element, interaction with transcriptional regulators (Igarashi and Ishihama, 1991; Ishihama, 1992; 1993; Russo and Silhavy, 1992). Point mutations in *E. coli* have identified individual residue of the α protein as potential contact sites for these activators. A significant proportion of these residues are conserved in *C. trachomatis*. *C. trachomatis* serovar L_2 possesses conserved residues at positions 291, 298, 303, 329 and 332; these correspond to the amino acid position 261, 265, 270, 296 and 299 of *E. coli* α protein, which are involved in its interaction with cAMP receptor protein, (Ebright, 1993). These results are important in view of the previous work from our laboratory showing the presence of a cAMP binding protein in *Chlamydia* (Kaul et al. 1990b). Further, mutation at position 289 of *E. coli* involved in anaerobic regulation is also conserved at position 322 of *C. trachomatis* (Lombardo et al. 1991). However, another mutation at position 311 is replaced with a nonconservative substitution. Mutations involved with the porin regulon have been mapped to the C-terminus of *E. coli* α protein and are not conserved in *Chlamydia* (Slauch et al. 1991). Whether the presence of a C-terminal tail in *Chlamydia* (Fig. 12) has anything to do with this non-conservation remains to be seen. Lys^{271} of *E. coli*, involved in interaction with the activators CysB, AraC and MelR, does not appear to be conserved in the chlamydial α protein (Thomas and Glass, 1991). However, the residue Leu^{323} of biovar L_2, corresponding to Leu^{290} of *E. coli* involved in bacteriophage P2 growth, is completely conserved (Fujiki et al. 1976).

Based on amino acid alignment, the chlamydial α subunit protein can be divided into four conserved regions (Fig. 12 and Table 4) similar to the chlamydial RNAP major σ subunit (Engel and Ganem, 1990). The functional importance of these conserved domains, if any, remains to be determined. However, significant conservation among α subunits was observed in the amino and carboxyl regions of the molecule. While the N-terminal portion of the *E. coli* α protein is involved in the assembly of the core RNAP, the 94 residues at the C-terminus are involved in interaction with transcriptional regulators

(Ishihama, 1992; Russo and Silhavy, 1992). Of the other regions, region II shows the least homology. The central region between conserved regions I and II revealed some major differences and exhibited a large gap during amino acid alignment. It is worth noting that the *C. trachomatis* α subunit protein contains an extended C-terminal tail of 18 amino acid residues (Fig. 12) similar to the one identified with the σ^{66} homolog (Lindahl et al. 1990; Koehler et al. 1990). Wichlan and Hatch (1993) recently identified an unusually A+T rich 5' upstream region in early upsteam ORF (EUO gene) that lacks a major σ consensus. These authors postulated that this (A+T) rich region may stimulate transcription by allowing an upstream regulatory protein to make contacts through DNA bending. Whether these extended tails allow RNAP to interact with additional factors such as the recently described (A+T) rich third recognition elements (UP elements) that are located upstream of -10 and -35 regions and play a role in the transcriptional process remains to be seen (Ross et al. 1993). It is, however, worth mentioning that there is nearly 70% bias towards A or T based on the calculated averages for the whole chlamydial genome (Gray et al. 1991).

Immunoblot analysis using rabbit antichlamydial α antibodies has shown chlamydial α subunit expression as early as 12 hours post infection in HeLa cells infected with EBs (Fig 13). A doublet comprising 42 and 40 kDa polypeptides was identified on the immunoblots. The 40-kDa band may represent degradation of the 42-kDa chlamydial α subunit protein, or alternatively, another cross reacting protein. Although protein synthesis could not be detected at an early time, it is reasonable to assume that EB harbors enough RNAP to initiate transcription of the early genes. These observations are further supported by immunoblot analysis carried out on infected cells grown in the presence of penicillin. Penicillin is known to arrest chlamydial growth, leading to abnormally large RBs (Barbour et al. 1982). While the steady state levels of chlamydial major outer membrane protein (expressed throughout its life cycle) and a 18-kDa eukaryotic histone H1-like protein (expressed late in its life cycle) are significantly reduced, no differences were observed in the expression levels of Ctrα subunit in penicillin treated cells. Immunogenic cross reactivity was also observed between chlamydial and *E. coli* α subunits of RNAP. These results suggest antigenic and structural conservation between these distantly related genera.

IV.4 Functional analysis of chlamydial RNAP α subunit and chimeric *E. coli* RNAP

I have examined the *in vivo* incorporation of chlamydial α subunit protein and its truncated variants into *E. coli* RNAP in order to study complementation of the chlamydial α gene product with its *E. coli* counterpart, assuming that the stoichiometry of RNAP is maintained by substitution of recombinant protein for native protein in these cells. While cells expressing the chlamydial α gene grew slowly in culture, a significant proportion of the chlamydial α subunit protein was found to be incorporated into *E. coli* RNAP (Fig. 14). Under similar conditions the N-terminal truncated α subunit lacking the region encompassing amino acids between 25-126 failed to associate with *E. coli* RNAP (Fig. 16). The finding that the truncated variant fails to associate with *E. coli* RNAP, in contrast to overexpressed full length chlamydial α subunit, reflects a specific interaction of chlamydial α subunit with *E. coli* RNAP. It also supports the view that the N-terminal region of the α subunit is required for the assembly of the multisubunit RNAP core enzyme in bacteria. The result of *in vivo* incorporation of the chlamydial α subunit protein into *E. coli* RNAP is also consistent with the previous demonstration that chlamydial r-protein L6 could be incorporated into functional ribosomes in *E. coli* in place of the host homologous r-protein (Gray et al. 1991).

The central region of the α subunit in *E. coli* is believed to contain a 13-36 amino acid unstructured and/or flexible linker which connects its N-terminal RNAP assembly domain and its C-terminal DNA-protein/protein-protein interaction domain (Ebright and Busby, 1995). With the construction of a deletion mutant lacking chlamydial central regions II and III, and having all four Cys residues intact, identification of a 27-kDa immunoreactive band (Fig. 17) from cells harboring plasmid pCTAdC demonstrates expression of central-truncated chlamydial α protein with *E. coli* RNAP. These experiments demonstrate (i) the stable expression of whole and truncated chlamydial polypeptides in *E. coli*, (ii) the incorporation of the chlamydial α subunit into *E. coli* RNAP, (iii) the failure of the truncated variants to associate with *E. coli* RNAP, and (iv) an antigenic and structural conservation among the distantly related genera *Chlamydia*, *Bacillus*, and *Escherichia*.

A significant proportion of chlamydial α subunit protein from overexpressing cells was found to be incorporated into *E. coli* RNAP. Whether *in vivo* substitution generates a functionally active enzyme was addressed by isolating *E. coli* RNAP containing heterologous α subunits and assaying for transcriptional activity. The interaction of chimeric RNAP (isolated from *E. coli* harboring recombinant plasmid pGEX-α) with glutathione-Sepharose strongly supports the incorporation of GST-α subunit fusion proteins into *E. coli* RNAP since the chlamydial α subunits directly purified from the glutathione-Sepharose column had RNAP activity and were able to transcribe the chlamydial MOMP gene. Further purification of the chlamydial α subunit from chimeric *E. coli* RNAP by glycerol gradient centrifugation was successful (Fig. 18). However, it is not clear whether the purified chimeric *E. coli* RNAP complex (subunit structure $\alpha_2\beta\beta'\sigma$) contains both heterologous α subunits or comprises one heterologous and one homologous α subunit. In any event, the purified chimeric complex initiated *in vitro* transcription from *E. coli* as well as chlamydial template DNA, but preferred to initiate transcription from chlamydial MOMP promoter (Fig. 19 A) probably because the chlamydial α subunit incorporated into *E. coli* RNAP has higher affinity for chlamydial promoters than *E. coli* pUC promoters. These results support the notion that the chimeric RNAP is functionally active. The authentic *Chlamydia* cell free RNAP isolated from RBs almost specifically transcribes chlamydial MOMP gene (Fig. 19 C) rather then pUC genes, suggesting that chlamydial RNAP recognizes and transcribes its own genes with high efficiency. Interestingly, preincubation of the MOMP and pUC template DNA with free chlamydial α subunit protein significantly increased transcription efficiency for the chlamydial MOMP gene with both high and low concentrations of chlamydial RNAP (Fig. 19 D and F), compared with transcription in Figure 19 C and E. The increased transcription efficiency for MOMP gene was probably promoted through binding of the α subunit to the DNA promoter region and protein-protein contact with other transcription factors (see next). These studies support the role of α subunit in transcription stimulation and promoter recognition and also suggest that chimeric RNAP is functionally active in an *in vitro* system. Some increased transcription from pUC genes by free chlamydial α subunit is consistent with the observation that chlamydial RNAP is able to transcribe some non-chlamydial genes *in vitro* (Mathews et al. 1993). The findings that low concentrations of chlamydial RNAP failed to transcribe the MOMP gene efficiently probably suggests that chlamydial cell-free extract alone is specific but not efficient because the RNAP is not saturated with σ factor (Douglas et al. 1994). However, our results suggest that the activity of chlamydial RNAP from cell-free extracts is concentration-dependent.

Douglas et al (1995) have shown that chlamydial MOMP transcription is initiated from the P2 promoter *in vitro*. Sequence analysis of the chlamydial MOMP P2 promoter region showed that the MOMP P2 promoter has an AT-rich UP element positioned in the region between -40 and -60 (greater than 75% A+T rich) (Kaul et al. 1990a). The C-terminus of the RNAP α subunit has been shown to have the ability to bind DNA at the A+T rich third recognition element (UP element) in bacterial promoters. Figure 20 shows that the chlamydial α protein binds to a 680-bp MOMP promoter containing DNA fragment, which can probably be explained by chlamydial RNAP α subunit binding through DNA-protein interaction. This hypothesis is supported by evidence that free chlamydial α subunit does not bind to the control DNA fragment which contains a 550 bp MOMP coding region. This may represent an alternate mechanism of enhanced transcription from chlamydial promoters lacking a consensus -35 region. This possibility is intriguing considering that the chlamydial α subunit contains an extended tail that may allow it to interact with elements that are even farther than *E. coli* UP elements. Further identification of the specific protein-DNA interaction region in this 680-bp fragment is in progress.

In *E. coli*, the *S10-spc-α* r-protein operons are located at position 72 minute on the chromosome map, while the genes for the β, β', and σ^{70} subunits are located at map positions of 90 and 67 minute (Bachmann, 1990). Using *Not*I, *Sgr*AI and *Sse*8387I (Fig. 21 A and B), I have mapped the *S10-spc-α* operons of *C. trachomatis* serovar L_2 based on the physical map published by Birkelund and Stephens (1992). The chlamydial *S10-spc-α* ribosomal protein operons are located within the region between the genes for the β, β' subunits and for σ factor 66 (Fig. 22), although the precise location on the *C. trachomatis* chromosome is still unclear. The order of the genes for the RNAP β, β', α subunits and housekeeping σ factor in *C. trachomatis* serovar L_2 is similar to that in *E. coli*. Previous reports have clearly demonstrated the eubacterial origin of *Chlamydia*, based on its 16S rRNA gene comparisons (Weisburg et al. 1986). The results presented in this dissertation have further substantiated these findings. Our results support high order operon conservation and gene localization among chlamydial r-proteins when compared to other bacterial species. These results are not surprising, given the paramount importance of ribosomes in protein synthesis.

The *spc* and α operons are the first reported complete chlamydial rprotein gene operons, representing an important step towards a better understanding of the mechanisms of chlamydial protein synthesis. It may also contribute new knowledge of the mechanism of action of antibiotics and antimicrobial resistance in *Chlamydia*. Moreover, characterization of chlamydial r-proteins may assist the development of new and powerful agents for fighting chlamydial infections.

The SecY protein is an integral membrane protein which occupies a central role in protein translocation, interacting directly or indirectly with cytoplasmic secretion factors and signal peptides of exported proteins. The mechanism and machinery of Sec-dependent protein translocation in *Chlamydia* are far from established since SecY is the only Sec protein reported to date in *Chlamydia*. Further work will be necessary focusing on immunoprecipitation of the chlamydial SecY complex using anti SecY antibodies to identify new Sec protein components, and subsequently their genes. Characterization of chlamydial Sec-dependent protein translocation mechanism may help to elucidate the morphological and functional effects of penicillin on *C. trachomatis* inclusions.

The cloning and characterization of the chlamydial α subunit represents a most important step towards understanding chlamydial RNAP holoenzyme, along with its characterized β, β' subunits and σ factor. Given the fact that *Chlamydia* is an obligate intracellular parasite and there are no conventional gene transfer techniques available for chlamydial gene manipulation, our functional chimeric RNAP provides a unique approach to purify RNAP without contamination from host counterpart molecules. It also furnishes an opportunity to perform *in vitro* transcription assays, specifically for chlamydial genes. Further work on the chimeric RNAP with different σ factors (*E. coli* σ^{70} and *Chlamydia* σ^{66}) to identify the precise recognition of different promoters and transcripts is currently in progress. As this knowledge proceeds, we will approach the goal of manipulating chlamydial genes *in vitro* without the need to purify chlamydial RNAP. This development would simplify the ability to perform chlamydial genetic studies such as *in vitro* transcription/translation assays, specific chlamydial promoter identification and *in vitro* synthesis of RNA probes.

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VI. Appendix

Amido black stain

45% methanol
10% acetic acid
0.1% amido black

BSG

0.15 M NaCl
2.2 mM KH_2PO_4
2.2 mM Na_2HPO_4
100 ug/mL gelatin

Coomassie Blue stain

0.25% Coomassie Brilliant Blue R-250
45% methanol
9% acetic acid

DNA buffer

10 mM Tris-HCl pH 7.8
5 mM NaCl
0.1 mM EDTA

DNA elution buffer

0.5 M ammonium acetate
10 mM $MgCl_2$
1 mM EDTA

DNA sequencing polyacrylamide gels:

4%- 50% urea
60 mM TEB
10% stock acrylamide

6%- 50% urea
60 mM TEB
15% stock acrylamide

Fill-in labelling reaction buffer (10 x)

0.33 M Tris-acetate pH 7.9
0.66 M potassium acetate
0.1 M magnesium acetate
5 mM DTT
0.1% BSA

Final sample buffer (FSB)

6.25 mM Tris-HCl pH 6.8
2.5% SDS
12.5% glycerol
0.01% bromophenol blue
0.5% β-mercaptoethanol

L broth

1.0% bacto-tryptone
0.5%bacto-yeast extract
1.0% NaCl

M9 salts (2x)

45 mM $Na_2HPO_4 \cdot 7H_20$
45 mM KH_2PO_4
17 mM NaCl
37 mM NH_4Cl

Nick translation buffer (5 x)

0.25 M Tris-HCl pH 7.2
0.05 M $MgSO_4$
0.5 mM dithiothreitol [DTT]
0.025% bovine serum albumin (BSA)

Plasmid extraction:

Solution1 50 mM glucose
25 mM Tris-HCl pH 8.0
10 mM EDTA
5 mg/mL lysozyme

Solution 2 0.2 N NaOH
1% SDS

Random primer labelling reaction buffer (5 x)

250 mM Tris-HCl pH 8.0
25 mM $MgCl_2$
10 mM DTT
100 mM HEPES pH 6.6
0.2% BSA

REB buffer

40 mM Tris-acetate pH 7.8
20 mM sodium acetate
2 mM EDTA

SDS-PAGE running buffer

27 mM Tris
19.2 mM glycine
0.1% SDS
pH 8.3

SPG

0.25 M sucrose
10 mM sodium phosphate
5 mM L-glutamine

SSC (10 x)

1.5 M NaCl
1.7 M sodium citrate, pH 7.0

SOC

2% bacto-tryptone
0.5% bacto-yeast extract
0.05% NaCl
2.5 mM KCl
Adjust the pH to 7.0 with 5 N NaOH
10 mM $MgCl_2$
20 mM glucose

STETL

8% sucrose
0.5% Triton X-100
50 mM Tris-HCl, pH 8.0
50 mM EDTA
0.5 mg/ml lysozyme

TAE

40 mM Tris-acetate
1 mM EDTA

TBE

45 mM Tris-borate
1 mM EDTA

TE

10 mM Tris-HCl pH 8.0
1 mM EDTA

TEB (1M)

1 M Tris
20 mM EDTA
1 M Boric acid

TES

10 mM N-tris(Hydroxymethyl)methyl-2-aminoethane-sulfonic acid
0.87% NaCl
Adjust pH to 7.4 with 1 N NaOH

TDG

10 mM Tris-HCl, pH 8.0
1 mM EDTA
3 mM DTT
10% glycerol
10 mM $MgCl_2$
100 µg/ml PMSF
10 µg/ml pepstatin A

TGED

10 mM Tris-HCl, pH 7.9
5% glycerol
0.1 mM EDTA
0.1 mM DTT

TSA

50 mM Tris-HCl pH 7.4
150 mM NaCl

TSN

TSA + 0.1% nonidet P-40

Western transfer buffer

0.025 M Tris base
0.2 M glycine
20% methanol

YT broth (and agar)

0.8% bacto-tryptone
0.5%bacto-yeast extract
0.5% NaCl
(±1.1% agar)